PHYTOCHEMICAL INVESTIGATION AND BIOLOGICAL ACTIVITIES OF *SANICULA EUROPAEA* AND *TEUCRIUM DAVAEANUM*

Isolation and identification of some constituents of *Sanicula europaea* and *Teucrium davaeanum* and evaluation of the antioxidant activity of ethanolic extracts of both plants and cytotoxic activity of some isolated compounds.

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Abstract

The aim of this research was to investigate the phytochemistry of two species *Sanicula europaea* and *Teucrium davaeanum* which are traditionally used in treatment of wounds. Four compounds were isolated from the 80% methanolic extract of *S. europaea*; bis-(2-ethylhexyl) phthalate (**1**), palmitic acid (**2**), rosmarinic acid (**3**), saniculoside N (**4**). Compounds **1** and **2** were isolated for the first time from this species. The structure elucidation of the isolated compounds was on the basis of 1D, 2D NMR spectroscopy and mass spectrometry measurements. Two compounds were isolated from the crude glycosides extract of *T.davaeanum*; **6** is a phenylethanoid glycoside and **8** is an iridoid glycoside, from the data available these may be new compounds for which the names davaeanuside A and davaeanuside B are proposed respectively."

The total polyphenol content of *S. europaea L, T. davaeanum* leaves-flowers and *T. davaeanum* stem were found to be 5.0, 1.20 and 0.65 mg per 100 mg dried plant material respectively. A study of the antioxidant activity of the 50 % ethanol extracts of *S. europaea* and *T. davaeanum* showed that on a mg/mg basis *S. europaea* and *T. davaeanum* have approximately 5%, 8 % antioxidant capacity of Trolox respectively.

A study of the cytotoxic activity of davaeanuside A (**6**), iridoid glycoside (**7**), davaeanuside B (**8**) and saponin compound (**10**) isolated from the crude glycosides extract of *T. davaeanum* revealed that saponin compound (**10**) inhibited the growth of Hela cells by 50 % at 50 µg/ml, $P < 0.001$, but the other compounds did not show activities against the tested cell lines at 100 µg/ml. The results of this work provide some basis for the traditional use of these species in the treatment of wounds.

Key words

Sanicula, *Teucrium*, saponin glycosides, phenylpropanoids, phenolic acids, antioxidant, cytotoxicity

To my husband who was supporting me through all the four years of my study,

To the spirit of my father,

To my family.

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ABBREVIATIONS AND SYMBOLS

A/PR/8/34	Strain A/Puerto Rico/8/1934
A/Vic/1/75	Strain A/Vic/1/1975
AD	Atopic dermatitis
$ABTS^{\cdot+}$	2.2`-azinobis(3-ethylbenzothioazoline-6-sulfonic acid
AB	Alamar blue
ACOOH	Acetic acid
B/Lee/40	Influenza B/Lee/1940
BT	Blomia tropicalis
BAL	Bronchoalveolar lavage
BUN	Blood urea nitrogen
$CHCl_3$	Chloroform
CM	Centimetre
$CDCl_3$	Deuterated chloroform
^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{12}C	12 Carbon
CYPZE1	Cytochrome P450 ZE1
CP	Cisplatin
C_5D_5N	Deuterated pyridine
CH_3CN	Acetonitrile
C-18	Carbon-18 silica gel
CE	Crude extract
D_2O	Deuterated water
DEPT	Distortionless Enhancement by Polarization Transfer
DMBA	Dimethylbenz(a) anthracene
DF	Dichloromethane fraction
DMSO	Dimethyl sulphoxide
ES+	Electro-spray positive mode
ES-	Electro-spray negative mode
EPSRC	Engineering and Physical Sciences Research Council
EGCG	(-)-epigallocatechin gallate
EAF	Ethyl acetate fraction

EtOAc	Ethyl acetate
FAB(-)	Fast atom bombardment negative mode
FRAP	Ferric reducing antioxidant potential
FBS	Foetal bovine serum
F_{254}	F_{254}
G	Grams
GC	Gas Chromatography
GC-MS	Gas-Mass Chromatography
G 60 F_{254} nm	Slica gel pore size 60 A, absorb UV light at 254 nm.
HL-60	Human promyelocytic leukemia cells
HCL	Hydrochloric acid
H_2SO_4	Sulfuric acid
^1H-^1H COSY	Correlation spectroscopy
HMBC	Heteronuclear multiple bond correlation
HepG2	Hepatocytes
H	Hour
HO-1	Hemeoxygenase
HIV-1	Human immunodeficiency virus type 1
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid
^1H NMR	Proton nuclear magnetic resonance
HPIV-2	Human parainfluenza Virus type 2
H_2O	Water
IC_{50}	Half maximal inhibitory concentration
IL-8	Interleukin-8
ICBN	International Code of Botanical Nomenclature
I/R	Ischemia and reperfusion
kg	Kilograms
KOH	Potassium hydroxide
L	Litre
MS	Mass spectrometry
MDCK	Madin Darby Canine Kidney cells
MIC > 500	Minimum inhibitory concentration

Ml	Milliliter
Mg	Milligrams
MeOH	Methanol
mM	Millimolar
Min	Minute
MeOD	Deuterated methanol
MHz	Mega Hertz
$[M+H]^+$	molecular ion peak
m/z	Mass-to-charge ratio
Mm	Concentration
MEM	Minimum Essential Medium
mg/kg	Miligram/kilogram
MEHP	Mono-(2-ethyl-5-hydroxyhexyl phathalate)
MACO	Middle cerebral artery occlusion
NMR	Nuclear magnetic resonance
nm	Nanometer
N_2	Nitrogen
NaH_2PO_4	Mono-sodium hydrogen phosphate
Na_2HPO_4	Di-sodium hydrogen phosphate
(NASH)	Non-alcoholic steatohepatitis
Na	Sodium
PPCC	positive pressure column chromatography
PHPLC	Preparative high performance liquid chromatography
pyridine-D-5	Deuterated pyridine
PBS	Phosphate Buffered Saline
PTLC	Preparative Thin Layer Chromatography
PPM	Parts-per million
RNA	Ribonucleic acid
RT	Retention time
RF	Retention Factor
SV	Surface view
SD	Standard division
TS	Transverse section

TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor-α
UV	Ultraviolet
U/VIS	Ultraviolet–visible spectroscopy
^{o}C	Degrees Celsius
µg	Micrograms
µl	Microliter
µm	Micrometer

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1 Introduction

1.1 The use of natural products in drug discovery

Natural products, of animal, plant and mineral origin, have been the roots of therapy of human illnesses and infections. The use of medicine essentially dates back basically to the beginning of human culture. Modern therapeutics have progressed over centuries' through systematic research of scientists. Recently, the most significant base of prospective drug leads was natural products, secondary metabolites (Bhushan *et al*., 2004).

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The Egyptian pharmaceutical Ebers Papyrus (2900 B.C.) detailed over 700 plant based drugs for production of gargles, pills, infusions and ointments. The clay tablets found in cuneiform from Mespotamia (2600 B.C.) were the earliest records of natural products. They mentioned uses of oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh) which are traditionally used as cure to coughs, cold and inflammation. The Chinese Materia Medica Wu Shi Er Binb Fang (1100 B.C.; recipes for 52 prescriptions). While natural scientist and Greek philosopher, Theophrastus (back to 300 B.C.) is a collection of medicinal herbs and the Shennong Herbal (100 B.C.; recorded the uses of 365 drugs (Bhushan *et al*., 2004).

The Greek physician, Dioscorides (100 A.D.) and Tang Herbal (659 A.D.; 850 drugs) are all mentioned the uses of medicinal herbs (Bhushan *et al*., 2004).

The Western knowledge of treatments (including England, Ireland, France and Germany) was conserved in monasteries during Dark and Middle Ages, while the Arabs maintained the Greco-Roman knowledge and expanded the uses of their resources. Arabs were the first who privately owned pharmacies (8th century) (Bhushan *et al*., 2004).

Many glycosides containing plant species are used in traditional medicine. For example *Sanicula europaea* and *Teucrium davaeanum* were used in treatment of wound. Therefore the aim of this study was to isolate and identify the main constituents of crude glycoside extracts of both species and test the isolated compounds in treatment of wound.

1.2 The genus *Sanicle*

Sanicle is a genus of the family *Apiaceae* or *Umbelliferae* (Wiersema, 2007). Both names are approved by the ICBN (International Code of Botanical Nomenclature). The *Apiaceae* family is a large family with about 250 to 455 genera and between 3300 and 3700 species. It is generally distributed in the temperate region of both hemispheres, mostly in Eurasia and especially in Asia; 100 genera (ten endemic and 614 species of which 340 are found in China (Zipcodezoo.com, 2013, Marquina et al., 2001). 28 genera and 137 species are native in southern Africa. In North America there are 91 genera; of these, about 20 are found in Quebec.

The most characteristic features of this family is that the plants have hollow stems named umbellifers, a characteristic small, in addition to, they have inflorescence in the form of compound umbel, flowers with five petals, and white, yellow or pink not blue colour (Michael, 2010).

The species belonging to the *Apiaceae* family are herbaceous, biennial or perennial plants, although some grow rough stems and there are a few wooded, tree-like or shrubby species in temperate areas. They have substitute and usually compound leaves, frequently simple, often fern-like or feather-divided; the leaves extend at the base into a sheath that clasps the stem. The stems are hollow between the leaf-joints, often furrowed, The cluster crystal of calcium oxalate, which are found distributed in abundant frequency in the mesophyll. The idioblasts, which are rarely found, are composed of parenchymatous cells densely packed with microsphenoidal crystals of calcium oxalate. These present in the spongy mesophyll and are frequently damaged, so that the crystals are scattered when seen under the microscope. The acicular crystals of calcium oxalate which are found in underlying parenchyma in surface view of the leaf their appearance are regular (Lindley, 1836).

The genus *Sanicula* has about 40 species worldwide, (Marquina *et al*., 2001). Table 1.1 shows the *Sanicula* species and subspecies with their botanical and common names (Lindley, 1836).

Table 1.1 *S. europaea* species and subspecies with their botanical names, common names and distribution.

No.	Species	Common name	Distribution
1	*S. apiifolia*	-	United States
2	*S.arctopoides*	Footsteps of spring	United States (California , Oregon , Washington , Mexico
3	*S. arguta*	Sharptooth Blacksnakeroot	United States (California) , Mexico
4	*S. artopodoides*	-	Temperate zone .
5	*S. astrantia*	-	Temperate zone
6	*S. astrantiifda*	-	-
7	*S. azorica*	-	Portugal , Turkey .
8	*S. bipinnata*	Poiso Sanical .	United States (California) , Mexico .
9	*S. bipinnatifida*	Purple Snakeroot .	United States (California , Oregon , Washinton) .
10	*S. brasiliensis*	-	-
11	*S. caerulescens*	Blue sanicle .	United States .
12	*S. canadensis*	Canadian Black snakeroot .	United States .
13	*S. canadensis var canadensis*	-	-
14	*S. canadensis var. grandis Fern .*	-	United States , Maxico .
15	*S. canadensis var. canadensis .*	Canadian Blacksnakeroot .	North America .
16	*S. canadenis var. genuine*	Canadian Blacksnakeroot .	United States .
17	*S. canadensis var. grandis*	Canadian Blacksnakeroot .	United States .
18	*S. canadensis var. marilandica*	Canadian Blacksnakeroot .	United States .
19	*S. canadian var. marilandica .*	Canadian Blacksnakeroot .	United States .
20	*S. capensis .*	-	-

Table 1.1 continued

21	*S. chinensis*	-	China , Japan .
22	*S. coerulescens* .	-	Qatar , Sudia Arabia , United Kindom , United States .
24	*S. crassicaulis*	Pacific Blacksnakeroot.	United States , Canada , Chile , Mexico .
25	*S. crassicaulis Var. crassicaulis*	Pacific Blacksnakeroot.	United States .
26	*S. crassicaulis Var. menziesii*	Western Snakeroot .	United States (Oregon , Washinton) .
27	*S. crassicaulis Var. tripartite*	Pacific Blacksnakeroot.	North America , United States .
28	*S. costata*	-	-
29	*S. crassifolia*	-	Chile .
30	*S. deserticola*	-	Mexico
31	*S. dielsiana*	-	-
32	*S. diversiloba*	-	-
33	*S. elata*	-	China , Japan , Cameroon , Equatorial , Indonesia .
34	*S. elongata*	-	-
35	*S. epipactis* .	-	-
36	*S. erythrophylla* .	-	-
37	*S. europ*	-	-
38	*S. europaea L.*	European Sanicle .	United States , Austria , Azerbaijan , Finland , France , Germany , Creece , Hungary , Ireland .
39	*S. europaea elata*	European Sanicle	-
40	*S. europea*	-	Norway , Spain , Sweden .
41	*S. floridana*	-	-

Table 1.1 continued

42	*S. floridana Var. Canadensis*	-	United States .
43	*S. giraldii*	-	-
44	*S. giraldii Var. ovicalycina*	-	-
45	*S. graveolens*	Western Snake-Root	United States , Canada , Chile , Mexico .
46	*S. gregaria*	-	-
47	*S. hacquetioides*	-	-
48	*S. henryi*	-	China .
49	*S. hermaphrodita*	-	United States .
50	*S. hoffmanii*	-	United States .
51	*S. hoffmannii*	Hoffmann's Blacksnakeroot	United States .
52	*S. howelli*	-	-
53	*S. ichangensis*	-	-
54	*S. javanica*	-	Indonesia .
55	*S. kaiensis*	-	-
56	*S. kauaiensis*	Kauai Blacksnakeroot	United States .
57	*S. kurilensis*	-	-
58	*S. laciniata*	Coastal Black snake root	United States , Mexico .
59	*S. lamelligera*	-	China . Japan , Taiwan
60	*S. liberta*	-	United States (Colorado , Florida) , Argentina , Colombia , Ecuador , Guatemala , Guinea .
61	*S. macrorrhiza*	-	-
62	*S. marilandica*	Maryland Black Snakeroot	Canada , Mexico , United States .
63	*S. marilandica Var. borealis*	Black Snakeroot	-
64	*S. marilandica Var. Canadensis*	Black Snakeroot	-

Table 1.1 continued

65	*S. maritima*	Adobe Snakeroot	United States .
66	*S. mariversa*	-	United States .
67	*S. marylandica*	-	United States .
68	*S. menziesii*	-	United States .
69	*S. menziesii Var. foliacea*	-	-
70	*S. menziessii Var pedata*	-	-
71	*S. mexicana*	-	Guatemala .
72	*S. montana*	-	-
73	*S. moranii*	-	Mexico .
74	*S. nanchuanensis*	-	-
75	*S. natalensis*	-	-
76	*S. nemoralis*	-	-
77	*S. nevadensis*	-	-
78	*S. nevadensis Var. glauca*	-	-
79	*S. nudicaulis*	-	-
80	*S. obtuse*	-	-
81	*S. ordorata*	Clustered Black Snakeroot	United States , Canada , Mexico .
82	*S. officinalis*	-	-
83	*S. officinarum*	-	-
84	*S. orthacantha*	-	China , Viet Nam .
85	*S. orthacantha Var. Costata*	-	-
86	*S. oviformis*	-	-
87	*S. palmate*	-	-
88	*S. patagonica*	-	-

Table 1.1 continued

89	*S. peckiana*	Peck's Blacksnakeroot	United States .
90	*S. pengshuiensis*	-	-
91	*S. petagnioides*	-	Taiwan .
92	*S. potaninii*	-	-
93	*S. purpurea*	Purple flower Blacksnakeroot	United States (Hawaii) .
94	*S. rubriflora*	-	Japan .
95	*S. sandwicensis*	Hawaii Black snakeroot	United States (Hawaii , south Georgia , South sandwich Islands .
96	*S. satsumana*	-	-
97	*S. saxatilis*	Devil's Black Snakeroot	United States .
98	*S. septentrionalis*	-	-
99	*S. serpentine*	-	United States .
100	*S. serrata*	-	China .
101	*S. smallii*	Small's Black Snakeroot .	North America Mexico .

1.2.1 *Sanicula europaea L.*

S. europaea root stock (underground stem from which every year's new stalks grow up) is shortly creeping and fibrous, with a small number of thick brownish scales at the top. The stem erect 8 inches to 2 feet high, often leafless or with a single leaf. The radical leaves are on stalks 2 to 8 inches long, the leaves themselves palmate three to five partite, the leaves are heart shaped at the base near the stalk and toothed like a saw. The flowers are in umbels, each little collection forms a semi-circular head. The umbel is irregular, the flowers pinkish-white, they may flower in May or June and are succeeded in August by spherical seeds which are covered with prickles, the plant is glabrous and clear green, the leaves paler beneath and the stems often reddish (Grieve, 1998).

Figure 1.1 and figure 1.2 shows the flowers and leaves in *S. europaea* respectively (Blaich, 2013a). Figure 1.3 shows distribution of *S. europaea* in the world (Zipcodezoo.com, 2013).

Figure 1.1 Flowers of *Sanicula europaea L* (Blaich, 2013b) .

Figure 1.2 Leaves of *Sanicula europaea L* (Blaich, 2013b).

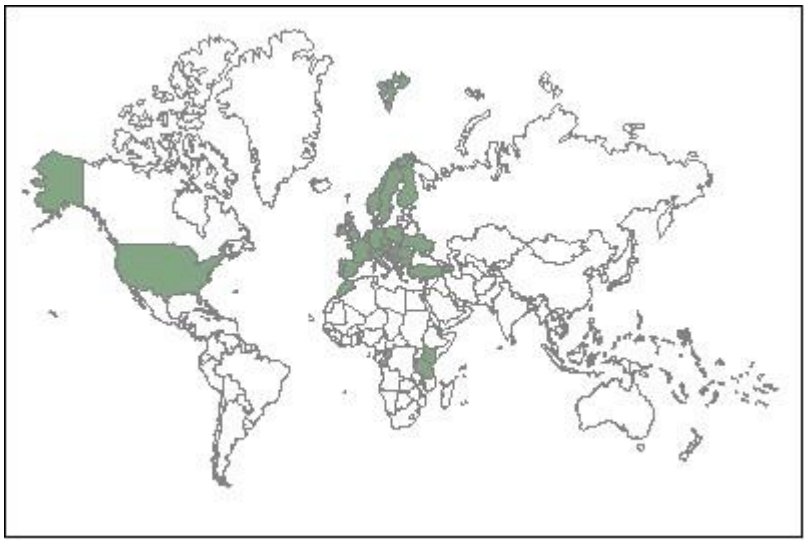


Figure 1.3 Distribution of *S. europaea L.*

1.2.2 Distribution of *S. europaea* in the world

S. europaea is found in the following countries; Austria, Azerbaijan, Belgium, Bosnia and Herzegovina, Bulgaria, Burundi, Congo, Czech Republic, Denmark, Equatorial Guinea, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Kenya, Morocco Netherlands, Norway, Poland, Portugal, Romania, Serbia and Montenegro, Slovenia, Spain, Sweden, Switzerland, Tanzania, Turkey, Ukraine, United Kingdom, United States (Zipcodezoo.com, 2013)

1.2.3 Traditional uses of *S. europaea .*

1.2.3.1 Use of *S. europaea* for wound healing

The Middle Ages were a time when several treatments for wound healing were vigorously used. Books remaining from this age and much later

argue in careful detail, the preparation and use of many plants specifically for wound healing. For example *Sanical* is a plant that is generally stated which is known today as *S. europaea* (Corke, 1912).

People in the Middle Ages used to say of *Sanicle* , “Celui qui Sanicle a De mire affaire il n’a” which means “He that hath *Sanicle* needeth no surgeon”. In reference to its medical virtues the name comes from the Latin word sano (I heal or cure). In the Middle Ages there was not one system approved for the naming of plants and spelling was not constant. This plant has many names. Such as, senikyl, senicle, senigle , wodemerche , wodeworte and siwariswort. This led the identification in the manuscripts a problem but the plant certainly grew and still grows in England today (Corke, 1912).

Many medical works record the use of S*. europaea* although when it was first introduced is unknown. The work written by Lanfranchi who is one of the well-known Italian surgeons of the Middle Ages, is the earliest example. He trained in thirteenth century Paris (Corke, 1912).

“Another powder is the viscous juice from the root of mallow, which some individuals called *Sanicle*, while other called ungaria, it is prepared by wash and dry the root in oven in such a method it does not burn, then grind it into a powder and apply a good amount to the hollow type of ulcer. Because of its inborn properties it encourages new tissue formation, either used by itself or mixed with other things” (Corke, 1912).

Many medical receipts that have been found in manuscripts from the thirteenth century mentioned the use of *S. europaea* . For instance, *S. europaea* is mentioned in the ˝ Lettre d’Hippocrate ˶ which was the most significant group of vernacular medical receipts before 1300 (Tony, 1994). It is also found in the versified receipts known as the ˝ Physique rime ˶ where sanicle is stated in a receipt for staunching the blood of wounds and in another for the therapeutic of wounds (Tony, 1994)

In the fifteenth centry *S. europaea.* was still being used in many treatments. For example, the ˝ Liber de Diversisor Medicinis ¨ which is a collection of remedies for various ailments written somewhere between 1422 and 1454 , point out *S. europaea* on many occasions (Ogden, 1938).

In an unpublished manuscript of 1446 *S. europaea* is mentioned in a number of recipes. For example “Also, through the use of certain drinks you can determine whether the wounded man shall live or die. For if he coughs it up again it is a sign of death and if he keeps it down it is a sign of life. One such drink is this one. Take 3 penny weights of bugle and *S. europaea* and crush them and mix them with 6 spoonfuls of old stale ale. Filter it and give it to the patient to drink” (Thomas, 1446).

“A good syrup for wounds..... or take equal quantities of herb John , herb Robert , Piggle , Bugle , Sanicle , Millifolie , Consolida major / minor , Plantain , Avence , use the juices ” (Thomas, 1446).

As late as 1794 an English dictionary was still able to comment upon it’s healing properties. “*S. europaea* – an evergreen plant , that grows wild in woods on hilly grounds , and is esteemed for it’s great vulnerary quality ” (Thomas, 1794). Where vulnerary = the proper cure of wounds and ulcers (Kanzaki et al., 1998).

1.2.4 Other uses of *S. europaea*

The uses of *S. europaea* as a medicinal treatment have been found in manuscripts dating as far back the 13th century (Grieve, 1998). It uses have been reported in treatment of blood disorders in combination with other herbs, as an internal remedy for chest and lung complaints, chronic cough and catarrhal infection, Inflammation of the bronchi, spitting of blood and infection of the pulmonary tissues, as an astringent gargle in sore

throat, in scald head of children and all cases of rash, for plentiful bleeding from the lungs, bowel and other internal organs and for examination dysentery.

1.2.5 Albarello drug jar for *Sanicle* , Italy , 1601 – 1800

Figure 1.4 shows a drug jar used to stock syrup of *Sanicle* which in Latin was called (Sciroppo di Sannicola). The syrup was composed of a mixture of *Sanicle* and sugar, this syrup was used to treat the inner ulcers, particularly in kidneys and bladder (museum.org.uk, 2013).

Figure 1.4. Jar used for syrup of Sanicle.

1.2.6 Phytochemistry of *S. europaea*

On reviewing the literature on *S. europaea* it was found that it contains many classes of compounds including saponin glycosides, tannins, volatile oils and mucilage-metabolic products, a summary of reported data is detailed below.

1.2.6.1 Volatile oil

Identification of the essential oil contents of *S. europaea* prepared by the hydrodistillation method from leaves of *S. europaea* was carried out by Pavlovic *et al* (2006). The plant was collected from two diverse regions, Montenegro and Serbia. The GC (Gas Chromatography) and GC-MS (Gas-Mass Chromatography) of the volatile oils revealed that the oils were composed mainly of sesquiterpene hydrocarbons (59.5-63.8%). The main constituents were β-selinene (40.4-44.2%), cayophyllene oxide (17.9-19.2) and α-selinene (3.7-4.0%), their structures are shown in Figure 1.5 (Pavlovic *et al*., 2006).

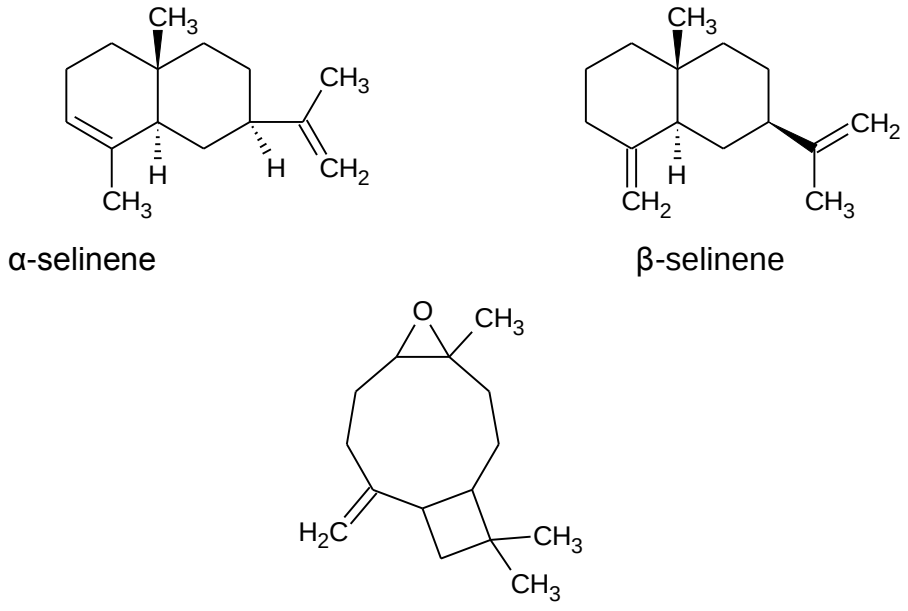


Figure 1.5. α-selinene, β-selinene and caryophyllene oxide.

1.2.6.2 Saponin glycosides

Arda *et al*. (1997) isolated a new triterpene saponin glycoside,21-β-(angeloyloxy)-3-*O*-[β-D-arabinopyranosyl(1-4)-β-D-glucopyranosyl(1-3)-β-

D-glucuronopyranosylpropyl-ester-3β,15,16,22α,28β-pentahydroxy-Δ^{12}–oleanene named saniculoside-N from *S. europaea*, Figure 1.6 (Arda *et al*., 1997).

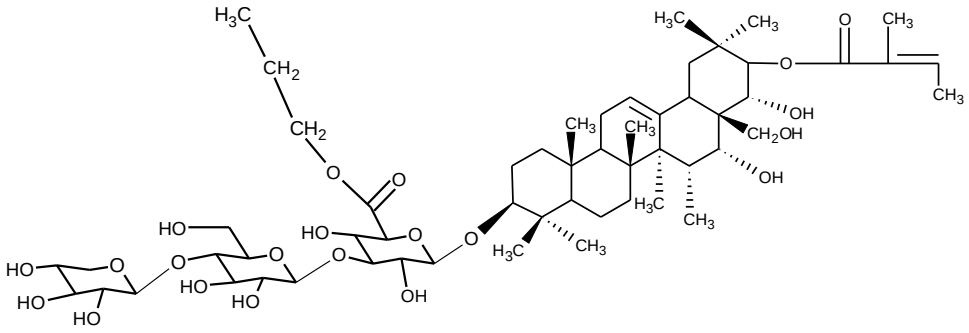


Figure 1.6. Saniculoside N.

Saniculoside R-1 Figure 1.7 is another triterpenoid saponin from *S. europaea* was isolated by Schopke *et al* (1998). The structure was elucidated from NMR and MS data as 21-*O*-[2-methyl butanoyl]-3β,15α,16α,21β,22α,28-hexahydroxyolean-12-ene-3-*O*-[α-L-arabinopyranosyl(1-3)]β-D-glucopyranosyl(1-2)-β-D-glucuronopyranoside (Schopke *et al*., 1998).

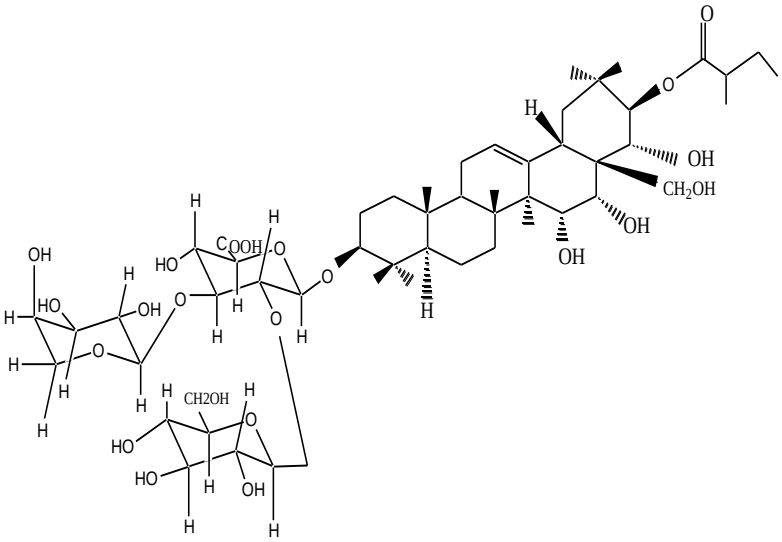
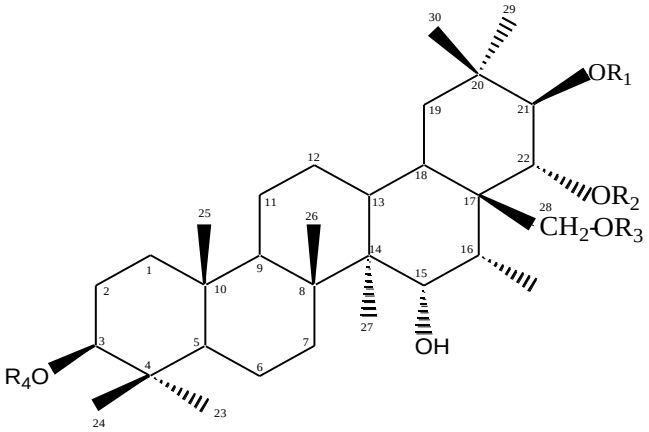


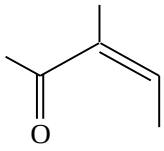
Figure 1.7 Saniculoside R-1.

Saponins have been found in some other species of *Sanicle* for example *Sanicula elata Ham. Var. chinesis Makino.* Matsushita *et al.* (2004) isolated eleven new oleanane type triterpenoid saponins, saniculasaponins (I-XI) and the known saponin sandrosaponin (IX) Figure 1.8 from the methanol extract of the total plant of *Sanicula elata Ham. Var. chinesis Makino*. The new compound structures were elucidated on the base of chemical and spectroscopic data (Matsushita *et al*., 2004).



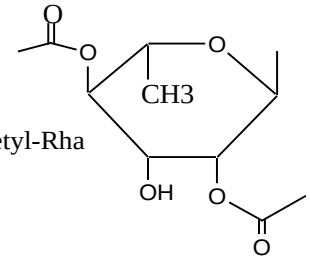
R_1	R_2	R_3	R_4
acetyl	angeloyl	H	-GlcA$^{3}_{2}$Gal, Glc
acetyl	angeloyl	H	-GlcA$^{3}_{2}$Ara, Glc
angeloyl	acetyl	H	-GlcA$^{3}_{2}$Ara, Glc
angeloyl	H	acetyl	-GlcA$^{3}_{2}$Ara, Glc
2,4-diacetyl-Rha	angeloyl	H	-GlcA$^{3}_{2}$Gla, Glc
2,4-diacetyl-Rha	angeloyl	H	-GlcA$^{3}_{2}$Ara, Glc
3,4-diacetyl-Rha	angeloyl	H	-GlcA$^{3}_{2}$Gal, Glc
3,4-diacetyl-Rha	angeloyl	H	-GlcA$^{3}_{2}$Ara, Glc

angeloyl



acetyl

2,4-diacetyl-Rha



3,4-diacetyl-Rha

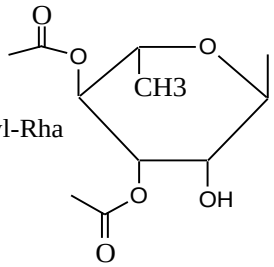


Figure 1.8 Saniculasaponins I-XI and sandrosaponin.

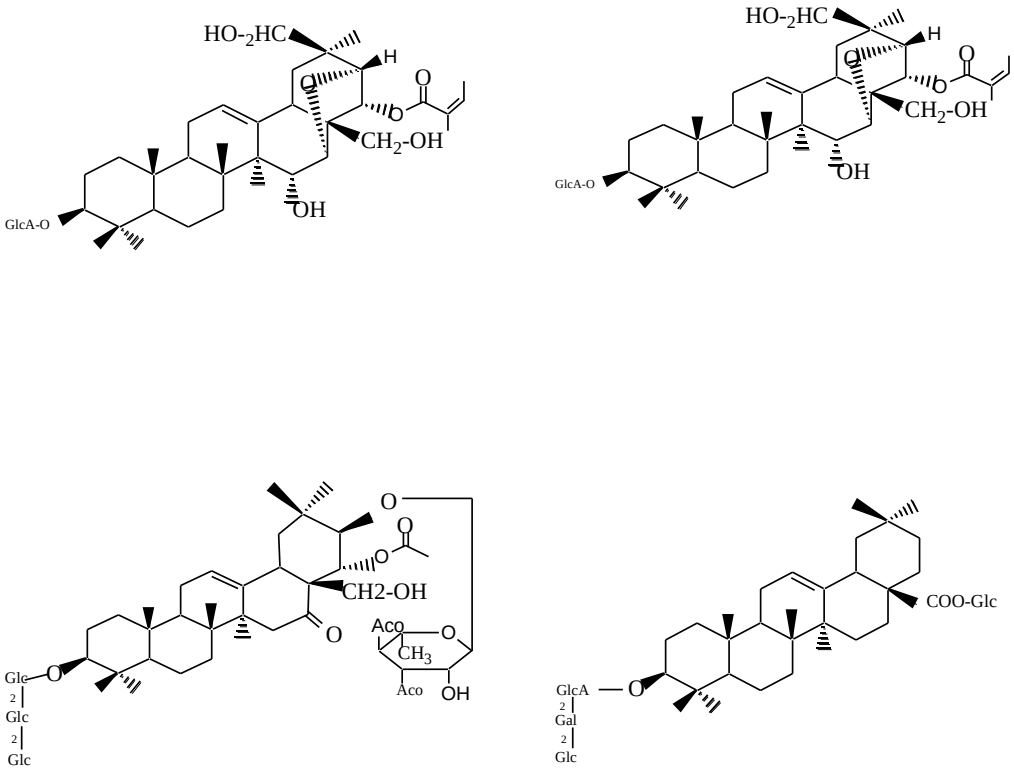
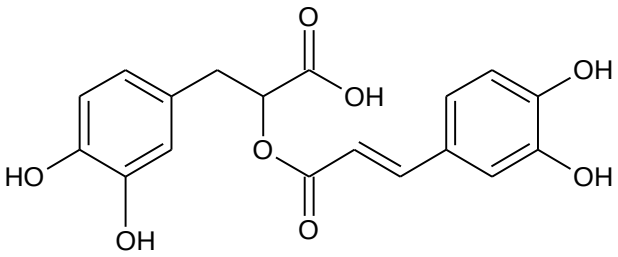


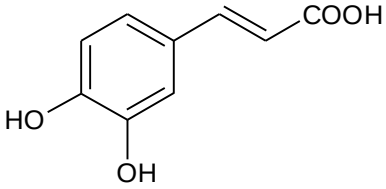
Figure 1.8. Continued.

1.2.6.3 Phenolic acids

The known phenolic acids, rosmarinic acid, and caffeic acid were isolated from *S. europaea* as major compounds Figure 1.9 (Arda *et al*., 1997).



Rosmarinic acid



caffeic acid

Figure 1.9. Rosmarinic acid and caffeic acid.

1.2.7 Pharmacological actions of *S. europaea*

The antiviral effect of aqueous extract of *S. europaea* leaf on influenza virus-infected cells was studied by Turan *et al.* (1996). Authors investigated the effect of aqueous extract of *S. europaea* on influenza virus growth in Madin Darby Canine Kidney cells (MDCK). Fractions I, II and III isolated from aqueous extract of *S. europaea* with Sephadex column chromatography were stated to be non-toxic toward MDCK cells at (100 µg/ml). The growth of influenza A/PR/8/34 (strain A/Puerto Rico/8/1934) was totally inhibited by these fractions, while that of influenza B/Lee/40 (strain B/Lee/1940) was not affected. Fractions II and III have

been shown not to have a direct virucidal activity on influenza A/PR/8/340 at (100 µg/ml) (Turan *et al*., 1996).

The effect of *S. europaea* aqueous extract on the virus multiplication was carried out by plaque inhibition assay. The results revealed that, plaque formation by influenza A/PR/8/34 was completely inhibited by fractions I. II, III at (100 µg/ml); thus, fraction IV was not effective at concentration of (50 µg/ml). According to plaque formation assay the same extracts were not effective against influenza B/Lee/40 multiplication. Influenza A/Vic/1/75 (strain A/Vic/1/1975) produced microscopic plaques in the presence of extract (no concentrations were mentioned) (Turan *et al*., 1996).

The RNA synthesis in vitro assay was also carried out. Preliminary experiments showed that the RNA polymerase activity of influenza A virus measured in vitro is inhibited by the addition of aqueous extracts (no concentrations were mentioned)
(Turan *et al*., 1996).

The antiviral activity of the aqueous extract of *S. europaea* leaf on the bacteria-bacteriophage system was studied by Turan *et al* (1996). They found that the plant extract inhibited the adsorption and/or penetration of phage infection without any toxic effect on *Escherichia coli* B2 host bacteria (no concentrations were mentioned). The high molecular weight fraction that eluted first from the gel filtration column was found to be accountable for this inhibition (Turan *et al*., 1996).

The antiviral activity of *S. europaea* aqueous extracts on multiplication of HPIV-2 (human parainfluenza Virus type 2) was studied by Karagoz *et al.* (1999). They prepared the aqueous extract from the leaf of the plant and a fraction isolated from the crude extract with gel filtration chromatography was shown to inhibit HPIV-2 reproduction without any toxic result on Vero cells. The acidic fraction (100 µg/ml) obtained from the crude extract of *S. europaea* leaf was found to be the most effective fraction in the plaque

inhibition assay at non-cytotoxic concentrations. Unfortunately, antiviral activity was not detected in the molecules purified from the crude ethanol extract of *S. europaea* (Karagoz *et al*., 1999).

Newton *et al.* in 2002 found that the herb part of *S. europaea* has weak antimycobacterial activity [Mycobactenium aurum MIC > 500 µg/ml, M IL smegmatis > 500 µg /ml] (Newton *et al*., 2002).

1.3 *Teucrium davaeanum* species

The studied species *T. davaeanum* belongs to the family Labiatae. The species grows in Wadi Telal, Sirte region, Libya, it is an endemic species. The aqueous extract of the *T. davaeanum* is used in folk medicine in treatment of wounds and as an antidiabetic agent. The reason of this study was to identify the main constituents of crude glycosides extract which might have wound healing activity, which may have potential in the treatment of wounds. Identification of *T. davaeanum* was reported in (section 2.1).

The Labiatae is a family of flowering plants, with approximately 220 genera and 4000 species widely distributed (Khoshnood-Mansoorkhani et al., 2010). Most of the species of the Labiatae are aromatic square stemmed herbaceous annuals or perennials, while some tropical species are trees. Many species of Labiatae have been used in medicinal practice, manufacture of perfumes, spices and as flavouring agents (Pettit *et al.*, 1966).

The Labiatae is represented by 22 genera and 65 species in Libya (Siddiqi, 1985). The genus *Teucrium* is a large genus with about 340 species worldwide (Bosabalidis, 2013).

1.4 Botanical description of *Teucrium davaeanum L*.

Suffrutose shrub, stem decumbent, subterete, much branched, younger branches covered with soft spreading branched hairs. Leaves ± sessile oblong - lanceolate, attenuate at base, tricrenate in the middle, obscurely nerved above, prominently nerved and densely bullate beneath, revolute margined, densely villose and woolly on both sides. Verticils forming dense ovate - subglobose terminal spikes. Calyx subsessile, membranous, tubular- campanulate, 10-nerved, teeth subequal, triangular, acute, Corolla pale yellowish, tube subincurved, villous, upper lip lobes

oblong, suberect, lower lip trilobed, lateral lobes oblong-lanceolate, middle one 1.5 longer than lateral ones, deebly concave and almost conduplicate. Stamens arcuate, filaments sparsely villose. Nutlets black, smooth and rugose (Siddiqi, 1985). Figure 1.10. illustrates *Teucrium davaeanum.*

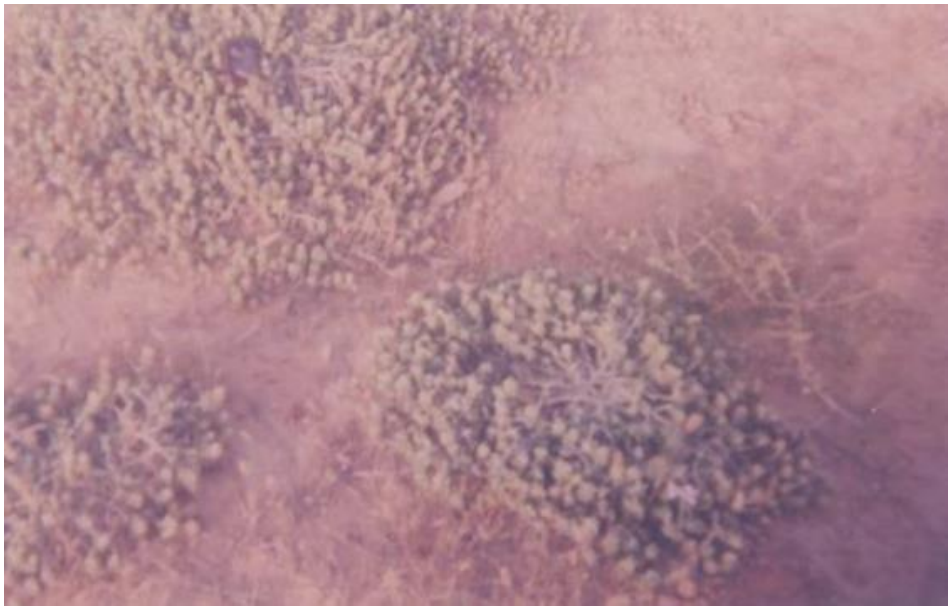


Figure 1.10 *Teucrium davaeanum*.

1.5 Phytochemistry of *Teucrium* genus

1.5.1 Volatile oils

Volatile oils were identified in many *Teucrium* species: *T. arduini* (Vukovic *et al*., 2011), *T. persicum* (Miri *et al.*, 2012), *T. orientale* subsp. Taylori (Amiri, 2010b) , *T. polium*, *T. polium ssp. Aurasiacum* (Jaradat, 2015), *T. libanitis* and *T. turredanum* (Blázquez *et al*., 2003), *T. cyprium ssp.*

Cyprium, *T. micropodioides*, *T. divaricatum* and *T. kotschyanum (*Arnold *et al*., 1991), *T. marum* (Ricci *et al*., 2005). The major constituents were monoterpenes and sesquiterpene: 1,4-Cadinadiene, limonene, germacrene-D and Beta-pinene. Figure 1.11 shows compounds identified in the volatile oils prepared from some *Teucrium* species.

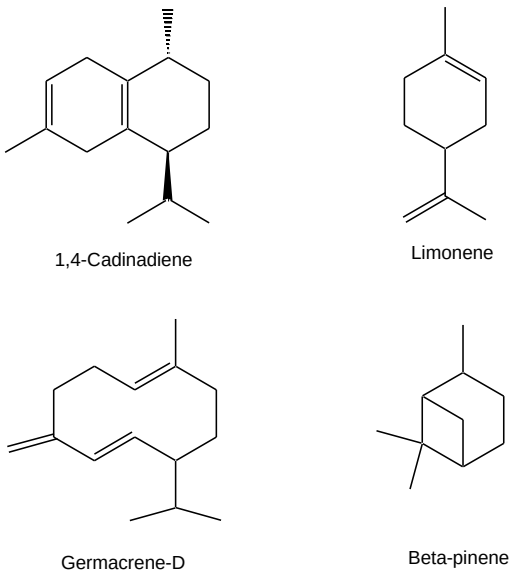


Figure 1.11 Shows compounds identified in the volatile oils prepared from some *Teucrium* species.

1.5.2 Diterpenoides compounds

Neo-clerodane and 19-nor-neo-clerodane diterpenoids compounds are very common in *Teucrium* genus. More than 200 neoclerodanes have been isolated and identified. Because of their biological activities, especially antifeedant activity, a large number of studies were carried out. Table 1.2 shows some diterpenoid compounds isolated from *Teucrium* species. Figure 1.12 shows diterpenoide compounds isolated from some *Teucrium* species.

Table 1.2 diterpeniode compounds isolated from Teucrium species.

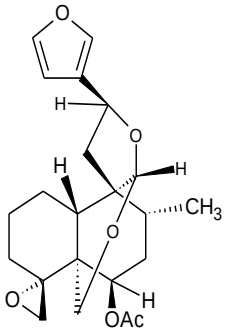
Species name	Diterpeniodes	References
T. abutiloides	Teubutilin A, B, Montanin C and 12-epiteupolin II.	(Ricci *et al*., 2005)
T. africanum	Tafricanin A, B.	(Hanson *et al*., 1982)
T. asiaticum	Teuflin, auropolin. Teucrasiolide acetylauropolin.	(Camps *et al*., 1987)
T. botrys	Teucvidirn, teuchamaedryn C, montanin D and teubotryn.	(María *et al*., 1986a)
T. barbeyanum	Teucrin A, F and G.	(Bruno *et al.*, 1985)
T. brevifolium	Teubrevins A and B. Teubrevinc C, D, E, F, G, H and I.	(Rodríguez *et al*., 1994)
T. bicolor	(12R)-epi-teuscordonin, montanin C, teucvin, teucrin H2 and teuscordonin .	(Labbe *et al*., 1989)
T. canadense	Teucvidin, teuflin), teucvin, 18-acetylmontanin.	(Bruno *et al.*, 1989)
T. capitatum	Lolin and 19-acetylgnaphalin	(Marquez et al., 1981)
T. chamaedrys	Teucrin A, teugin and dihydroteugin.Teucroxide. Teuchamaedrin C, 6α-hydroxy teuscordin and dihydroteugin. Teuchamaedryn A. Isoteuflidin, teuflidin, teucvin, teucvidin , teucrins F and teucrins G.	(Savona *et al.*, 1982). (Garcia-Alvarez *et al*., 1983). (Malakov and Papanov, 1985). (Lekehal *et al*., 1996). (Rodríguez *et al*., 1984)
T. chamaedrys ssp. Syspirense	Syspirensins A and Syspirensins B.	(Calis *et al*., 1996)
T. cubense	Eugarzasadone.	(Domíguez *et al.*, 1974)
T. flavum sub flavum	Teuflin and teuflidin	(Bruno *et al*., 1981)
T. flavum sub glaucum	Teuflin, teuflidin, teuflavin and teuflavoside.	(Savona *et al*., 1984c)
T. fragile	Teugin.	(Bruno *et al*., 1981)
T. creticum	Teucretol and 6,19-diacetylteumassilin.	(Savona *et al*., 1987) (Bruno *et al.*, 1992)

Table 1.2 Continued.

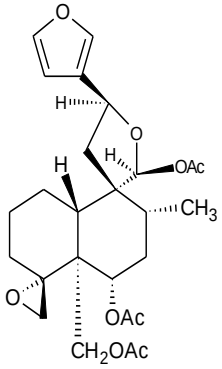
Species name	Diterpeniodes	References
T. gnaphalodes	Gnaphalin and 19-acetylgnaphalin. 12-acetylteugnapholodin.	(Savona *et al*., 1979) (María *et al*., 1985)
T. japonicum	Teuponin.	(Zhi-Daz *et al*., 1991)
T. kotschyanum	Teucvidin, teuflin, tuscorodin, 12-epiteucvidin, 12-epiteuflin, teukotschyn, montanin D and teucrin H_2.	(Simoes *et al*., 1989)
T. maghrebinum	12-epi-teucjaponin A, 12-epi-montanin D, 12-epi-montanin B, teusalvin C, teucjaponin A, montanin D, 19-deacetylteuscorodol and montanin B. Teukotschyn, 12-epi-teukotschyn, teughrebin and 12-epi-teughrebin.	(Bruno *et al*., 2000) (Bruno *et al*., 2001)
T. marum	Teumarin and acetyl teumarin.	(Savona *et al*., 1984b)
T. massiliense	Deacetylajugarin, Ajugarin, teumassilin, 6,19-diacetylteumassilin and triacetylteumssilin.	(Savona *et al*., 1984a)
T. montanum	Montanin A and B.	(Malakov *et al*., 1978)
T. montbretii subsp. Libanoticum	3β-hydroxyteututilin A, 12-epimontanin G, 20-epi-3, 20, di-O-deacetylteupyreinidin, 6-ketoteuscordin, teuscordinon, 6β-hydroxyteuscordin, montanin D, 3,20-di-O-deacetylteupyreinidin , montanin G, 3-O-deacetylteugracilin A.	(Bruno *et al.*, 2002)
T. Oliverianum	Teucrolivins A, B and C. Teucrolivins G and H. Teucrolins A, B, C, D F, G and E, 12-*O*-methylteucrolivin A, 12-O-methylteucrolin A.	(Bruno *et al*., 1991) (María *et al*., 1991) (Al-Yahya *et al*., 1993) (Al-Yahya *et al*., 2002)
T. oxylepis subs marianum	Isoteucrin, teucroxylepin, 12-O-acetylteugnaphalodin, diacetylteucroxylepin and teucjaponin A.	(Cuadrado *et al.*, 1991)
T. orientale	Teucrolivin A, B, C and H. 6-deacetyl-teucrolivin A, 8β-hydroxy-teucrolivin B.	(Bruno *et al.*, 2004)
T. pernyi	Teupernin D.	(Xie *et al.*, 1992)

Table 1.2 Continued.

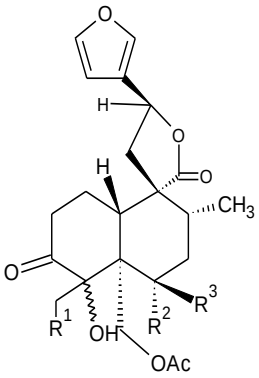
Species name	Diterpeniodes	References
T. pyrenaicum	Teupyrenone, teupyreinin, teupureinidin, teucrin P_1, 3β-acetoxyl-teupyreinin and gnaphalidin. Teupyrin A, B and teumassilin.	(García-Alvarez *et al*., 1982)
T. polium	Teupolin IV), teupolin V and teucrin P_1. Teulolins A and B. Teupolin III.	(Malakov and Papanov, 1983). (Bedir *et al.*, 1999) (Malakov *et al.,* 1982)
T. polium subsp aureum	Auropolin) and eriocephalin.	(Eguren *et al.*, 1981)
T. polium subsp capitatum	Picropolin, 7-deacetylcapitatin, picropolinol, 20-epi-isoeriocephalin and capitatin.	(Fernández *et al.*, 1985)
T. salviastrum	Teusalvin A, 18-acetyl teuscorodin, teuscorodin, teusalvin B ,teuscorodol, teusalvins D, E and F.	(María *et al*., 1986b)
T. scorodonia	Teuscorolide-2-hydroxyteuscorolide, teuscorodin and teuscorodonin.	(Marco *et al*., 1983)
T. scordium	2-keto-19-hydroxyteuscordin .	(Papanov and Malakov, 1985)
T. viscidum var miquelianum	Teucvidin, methyl teucvate and methyl teucvidinate.Teucvin and teufin.	(Node *et al*., 1981)
T. webbianum	Teuflidin, teucrin A, 2β-hydroxyteucvidin, teucvidin, teucvin, teuflin, teucrin H_4 and isoteuflidin.	(Savona *et al.*, 1986)
T. yemense	6β-O-acetyl-3β-hydroxy teucroxylepin, teucryemin, 19-acetyl teucryemin, 3β-19-O-diacetylteucryemin and teucryeminone.	(Sattar *et al.*, 1995)



Teubutilin A

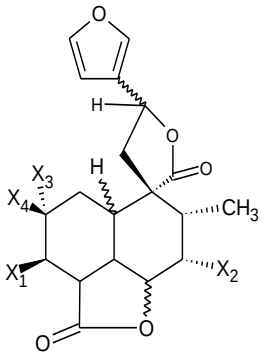


Teubutilin B

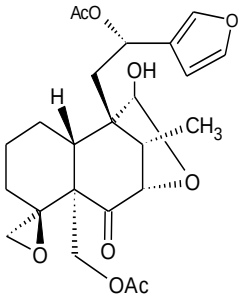


Tafricanin A $R^1 = Cl, R^2 = R^3 = O$

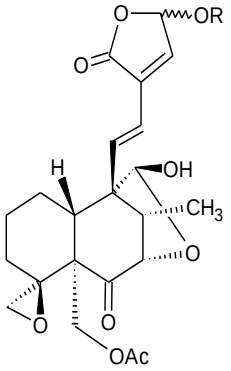
Tafricanin B $R_1 = Cl, R_2 = OAc, R_3 = H.$



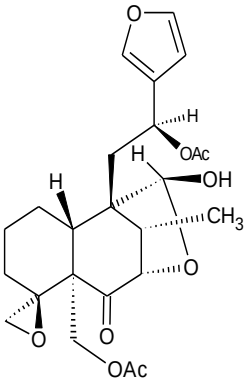
Teuflin



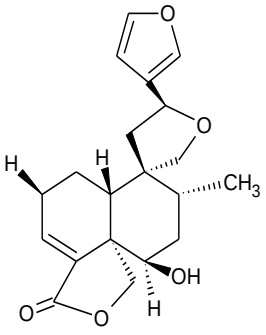
Auropolin



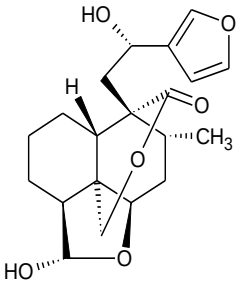
Teucrasiolide



Acetylauropolin



Teucvidirn

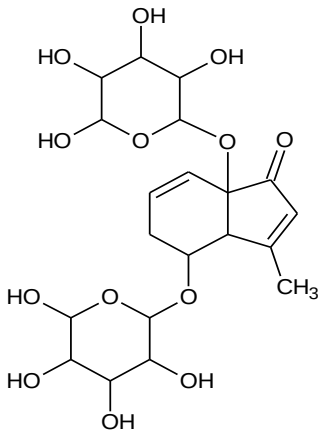


Teuchamaedryn C

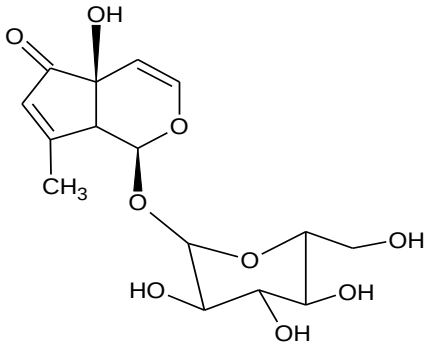
Figure 1.12 Shows diterpenoide compounds isolated from some *Teucrium* species.

1.5.3 Iridoid glycosides from *Teucrium* species

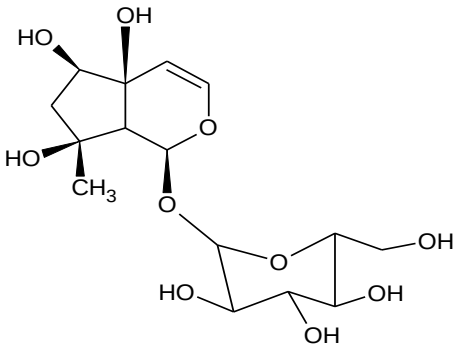
The iridoid glycosides harpagide and 8-O-acetyl harpagide are common in *Teucrium* species. The two compounds were reported in *T. polium* in addition to teucardoside and teuhircoside (Jaradat, 2015) (Rizk *et al*., 1986) . Acetyl harpagid and teucardoside were isolated from *T. arduini* L (Ruhdorfer and Rimpler, 1981). Figure 1.13 shows iridoid glycosides isolated from some *Teucrium* species.



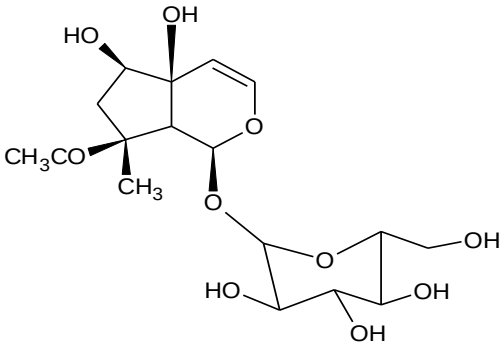
Teucardoside



Teuhircoside



Harpagide



8 - O - acetyl harpagide

Figure 1.13 Iridoid glycosides isolated from some *Teucrium* species.

1.5.4 Flavonoids

Kawashty *et al* (1999) isolated five flavonoids from the leaves and stems of *T. polium*. The compounds were named apigenin 7-glucoside, vicenin-2, luteolin 7-glucoside, apigenin 5-galloylglucoside and cirsimaritin (Kawashty *et al*., 1999).

Rizk *et al* (1986) isolated and identified luteolin, luteolin-7-O-glucoside and 6-methoxygen-kwanin from *T. polium.* Oganesyan (2007) isolated the eupatorin (5,3-dihydroxy-6,7,4`-trimethoxyflavone) from the aerial part of *T. orientale* (Oganesyan, 2007). Barberan *et al* (1985) isolated luteolin and apigenin from the aerial part of *T. gnaphalodes* (Barberan *et al*., 1985). Figure 1.14 **s**hows flavonoids isolated from some *Teucrium* species.

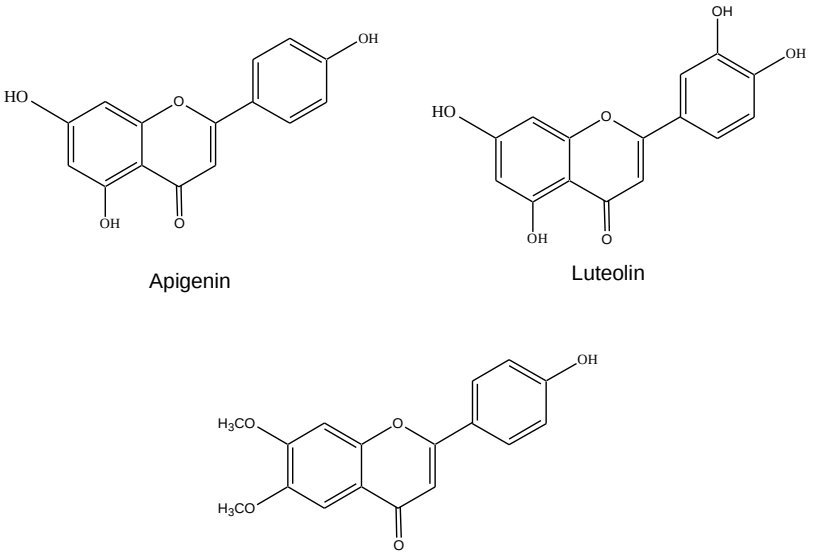


Figure 1.14 Shows flavonoids isolated from some *Teucrium* species.

1.5.5 Biological activities of some *Teucrium* species

Teucrium polium showed antidiabetic activity (Tatar *et al.*, 2012), *T. arduini* L. exhibit antioxidant and antimicrobial activities (Šamec *et al*., 2010), *T. persicum Boiss* showed antioxidant activity (Miri *et al*., 2012), *T. orientale* showed antioxidant activity (Amiri, 2010a). *T. chamaedrys* showed antioxidant and antimicrobial activities (Vlase *et al.*, 2014), hepatotoxicity (Lin *et al*., 2009), (Gori *et al*., 2011), treatment of wound (Chiej, 1984), treatment of digestive, respiratory disorders, abscesses and gout (Stankovic *et al.*, 2010).

1.6 Aim of the study

The overall aim of this work was to investigate the phytochemistry of two plant species: *Sanicula europaea and Teucrium davaeanum* and to determine their total phenol contents as well as evaluate their antioxidant and cytotoxic properties.

1.7 Objectives

- Prepare the plant extracts, using solvent extraction.

- Isolation of the major constituents using open column chromatography (CC), preparative chromatography (PC) and high performance liquid chromatography (HPLC).

- Elucidate the chemical structures of the isolated compounds using spectroscopic techniques (MS, 1D & 2D NMR).

- Determine the total phenol contents of 50 % ethanol extract of the two plants species via Folin-Ciocalteu's method. Evaluate the antioxidant activity of 50 % ethanol extracts of both plants using ABTS radical scavenging activity.

- Evaluate the cytotoxic activity of some isolated compounds from *T. davaeanum* according to the method published in (Fatokun et al., 2013).

2 Materials and Methods

2.1 Plant materials

Dried cut herb of *S. europaea* was supplied by the Herbal Apothecary Company (Leicester).

Teucrium davaeanum was collected from Wadi Telal. Sirt region, in April 2013 during the flowering stage, the plant was kindly identified by Dr Mohammed ElSherif, Biology Department, Faculty of Science, Garyounis University-Benghazi, Libya.

2.2 Solvents

The solvents (reagent grade and HPLC grade) were purchased from Fisher Scientific.

2.3 Chemicals

Chemicals were purchased from Sigma Aldrich Scientific unless otherwise stated.

2.4 Extraction procedures

2.4.1 Preparation of non-polar constituents of *S. europaea*

This procedure was carried according to the method of Alla *et al.* (2013). Dried and crushed plant material of *S. europaea* was defatted in a Soxhlet extractor at 50 ^{o}C using petroleum ether for 5 hours. The extract was dried over anhydrous sodium sulphate, filtered and evaporated *in vacuo* at 45 ^{o}C until dryness to yield a yellow material (4g) (Alla et al., 2013).

The petroleum ether extract was then dissolved in boiling acetone (300 ml) and left at room temperature for 24 hours. The solution was filtered and the precipitate washed first with cold acetone and then with cold chloroform:methanol (1:1) to yield a white precipitate (216 mg) of acetone insoluble fraction (fatty alcohols). The acetone soluble fraction (fatty acids + hydrocarbons) was evaporated to dryness (1.5 g) (Alla et al., 2013).

2.4.1.1 Preparation of hydrocarbons mixture

The acetone soluble fraction after evaporation in rotary evaporator (2.8 g) was saponified by refluxing with 60 ml 0.5 M methanolic KOH (5.6 g KOH in 100 ml MeOH) for 6 hours. The mixture was concentrated to about 30 ml and diluted with distilled water (40 ml). The unsaponifiable fraction was extracted with ether (3 × 100 ml). The combined ether extract was washed with distilled water, dried over anhydrous sodium sulphate and concentrated *in vacuo* to dryness to yield a yellow semisolid residue of unsaponifiable fraction (e.g. hydrocarbons mixture) (2.3 g).

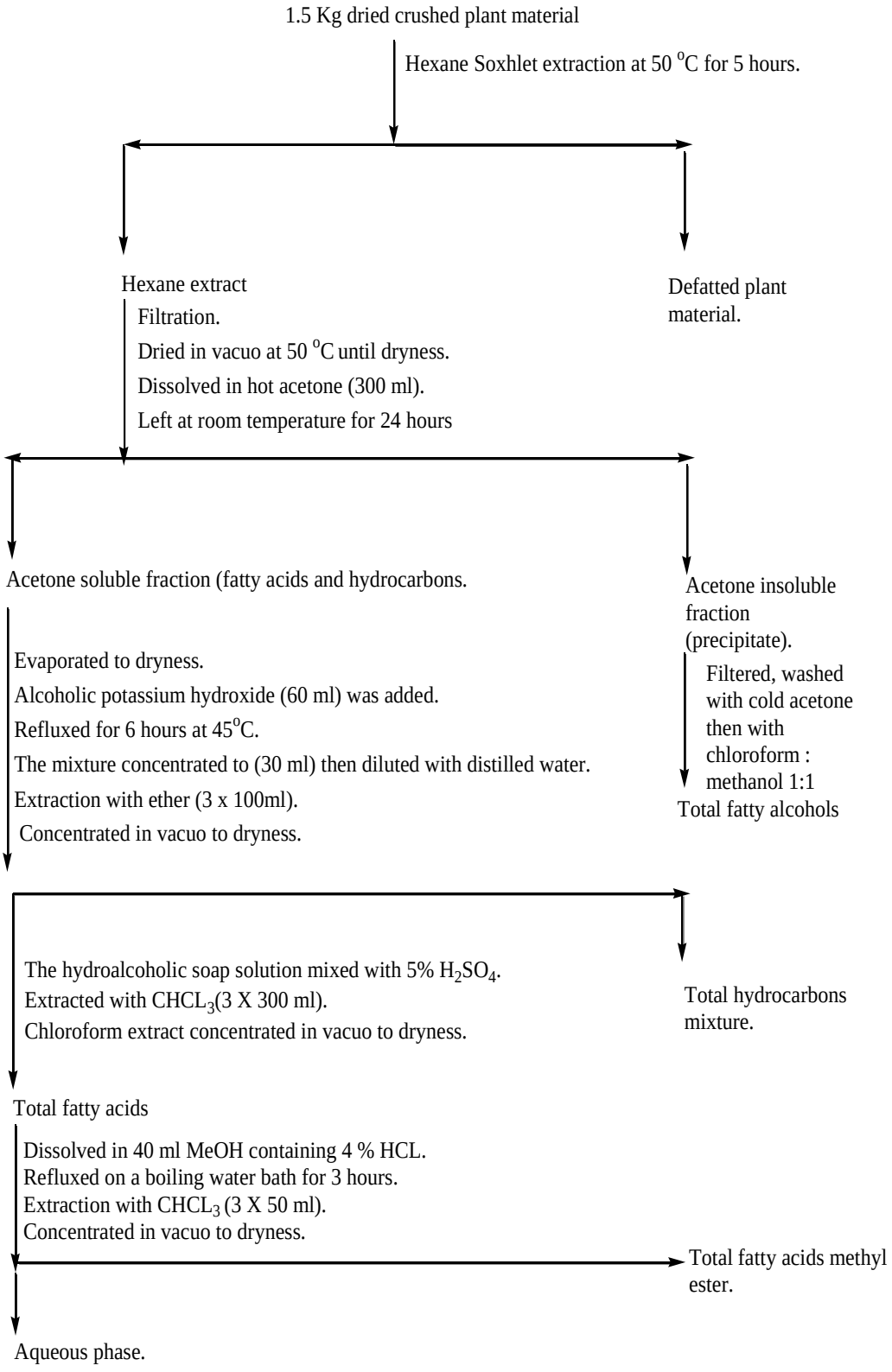


Figure 2.1. Extraction of non-polar constituents of *S. europaea*.

2.4.1.2 Extraction of glycosides from *S. europaea*

The defatted dried plant material (section 2.4.1) was Soxhlet extracted using 80% methanol at 45 ^{o}C for 5 hours. The solution was filtered using filter paper Whatman No 1. The methanol in the solution was removed using a rotary evaporator at 45 ^{o}C. The remaining aqueous solution was extracted several times with chloroform (3 × 250 ml), followed with ethyl acetate (3 × 250 ml) then butanol (3 × 50 ml).

The combined butanol layers were concentrated to dryness (using a rotary evaporator at 70 ^{o}C, methanol was added to reduce the boiling point of the butanol) i.e. forming a eutectic mixture. The dried butanol extract was then dissolved in methanol, and then dropped slowly into diethyl ether. The whitish precipitate obtained from the above procedure was filtered using a sintered glass Buchner funnel. The precipitate was dried under vacuum for 18 hours.

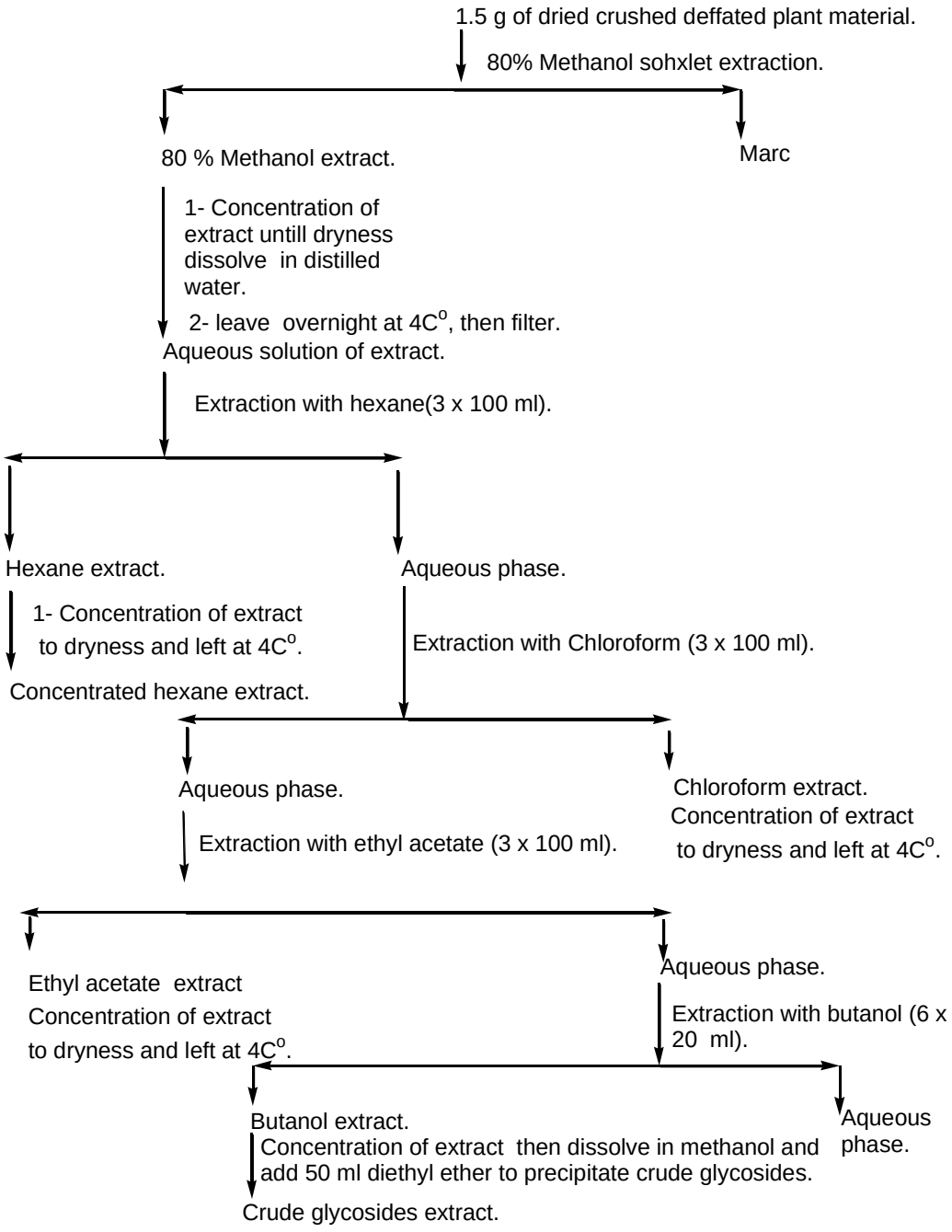


Figure 2.2. Extraction of crude glycosides of *S. europaea*.

2.4.1.3 Aqueous extraction

Dried and crushed plant material of *S. europaea* (10 g) was added to 200 ml of distilled water and the mixture was shaken strongly for 30 minutes, filtered and divided into two portions in thick walled pear-shaped flasks (100 ml each). The samples were frozen using dry ice acetone and freeze dried in the Institute of Cancer Research with the help of Dr Bradley.

2.5 Chromatographic Techniques

2.5.1 Normal phase silica gel chromatography

Fractionation of plant extracts was carried out using positive pressure column chromatography (PPCC). The column was packed with normal phase silica gel for column particle size 0.02-0.04 mm - 0.04-0.06 mm Silica gel for thin layer chromatography particle size 12 µm was also used. The mobile phase used was a gradient system of hexane:chloroform: methanol.

2.5.2 Reverse phase silica gel C-18 chromatography

Reverse phase silica gel C-18 type (LiChrospher ^{R} 100 RP-18E 12 µm) was used. The column (30cm length and 2.5 cm diameter). was packed with a slurry in methanol: water, 50: 50 and allowed to settle under gravity. The crude glycosides extract was dissolved in a minimum amount of methanol and added to the column; then it was continually eluted with the selected mobile phase. Fractions were collected with a volume ranging between 15-20 ml.

2.5.3 Preparative thin layer chromatography

Preparative chromatography of plant extracts was performed using two types of plates, thin layer chromatography (silica gel 60 F_{254} aluminum sheets 20 × 20 cm plates) and thick layer chromatography plates (silica gel 60 F_{254} glass plates 20 × 20 cm, 2000 microns). The mobile phase was Chloroform: Methanol: water 60:40:10 for both plates. Capillary tubes (5 µL) were used to apply the extract as a band across the plate; four applications were placed on each plate. The mobile phase was developed through the plate three times.

Plates were allowed to dry and covered with glass except for the edge. The edge was sprayed with vanillin sulphuric acid. The plates were visualised under UV light at wavelengths 254 and 375 nm. The bands were marked by pencil and collected using a small spatula. The combined bands were collected in small vials, dissolved in methanol, sonicated for 15 minutes and filtered. The methanol was evaporated using a rotary evaporator.

2.5.4 Gel filtration chromatography

Fractionation of plant extract was carried out using Sephadex LH20. The slurry was prepared in chloroform or methanol. Mobile phases used were Methanol (100%), Chloroform:Methanol gradient system, Methanol:water 50:50.

2.5.5 Thin layer chromatography (TLC)

Analytical (TLC) was performed using silica gel 60 F_{254} on aluminium sheets 20 x 20 cm, 0.25 mm layer (Merck Ltd). The resulted from fractionation of crude glycosides was applied on TLC by 2 µm, 5 µm

capillary tubes. The most commonly used mobile phase was chloroform: methanol:water (6:4:1) tanks with different sizes were used.

2.6 Gas chromatography-Mass spectrometry (GC-MS)

Instrument	AGILENT 7890 GC with 5975 EI/CI MSD.
Temperature program	Injector: 250°C, Detector: 300°C.
Oven program	initial temp 100°C for 0.5min, ramp 1: 5°C/min to 220°C, no hold, ramp 2: 10°C/min to 275°C hold 5 min.
Run time	35min.
Carrier gas	Helium at 1ml/min.
Column	Capillary column SGE BP5, length 25m, film thickness 0.5 µm, internal diameter 0.32mm.
Detector	Flame ionization detector.
Sample volume	1µl.

2.7 High performance liquid chromatography (HPLC)

2.7.1 Analytical High performance liquid chromatography (HPLC)

Model	Millipore Waters-510
Detector	Linear UVIS 200
Wavelength	254 nm
Loop	20 µl, 100 µl
Pump	510-Solvent system pump
Column	Reverse phase silica gel C-18 (10 × 100mm) Normal phase silica gel (10 × 225 mm)

2.7.2 Preparative high performance liquid chromatography (PHPLC)

Model	Perkin Elmer Series Autompter
Detector	Series 200 Diode Array Detector
Wavelength	(200, 290 and 330) nm
Loop	100 µl
Pump	Perkin Elmer Series 200 pump
Column	Reverse phase silica gel C-18

2.8 Spray reagents

2.8.1 Anisaldehyde reagent

0.5 ml anisaldehyde was mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid (Wagner, 2001).

2.8.2 Vanillin sulphuric acid reagent

2 g vanillin was dissolved in 100 ml of methanol; 2 ml of sulphuric acid were added (Wagner, 2001).

2.8.3 Ferric chloride reagent

Ferric chloride was prepared by dissolving 5 g of ferric chloride in 100 ml methanol.

2.8.4 Dragendorff's reagent

Solution A was prepared by dissolving 0.85 g of basic bismuth nitrate in 10 ml glacial acetic acid; 40 ml of distilled water was added. Dissolution may be encouraged by heating.

Solution B was prepared by dissolving 8 g potassium iodide in 30 ml of distilled water. Stock solution was prepared by mix equal volumes of solution A and B Dragendorff's reagent was prepared by mixing 1 ml of stock solution with 2 ml of glacial acetic acid and 10 ml of water.

2.9 Spectroscopic methods

2.9.1 Mass spectrometry

2.9.1.1 Electro spray ES+ and ES- mass spectrometry

The instrument used was a Micromass Quattro Ultima Spectrometer. Ionisation was carried out using electro spray (ES+) and (ES-). Samples were dissolved in methanol and infused at 10µl / min using a Harvard syringe pump.

2.9.1.2 Accurate mass spectrometry

Mass spectrometry data were acquired at the EPSRC UK National Mass Spectrometry Facility at Swansea University.

2.9.2 Nuclear Magnetic Resonance spectroscopy (1-D,2-D NMR)

These were recorded in one of the following solvents $CDCl_3$, MeOD, D_2O, pyridine-D-5. The instrument used was a Bruker Ultra Shield 400 MHz (^1H NMR spectra were recorded at 400.1300 MHz, ^{13}C NMR at 100.6126 MHz, DPX 400 or JEOL ECA 600 (^1H NMR at 600.1723 MHz, ^{13}C NMR at 150.9134 MHz).

3 Results and discussion, phytochemistry of *S. europaea*

3.1 Identification of the lipid fraction of *S. europaea*

Total lipid fraction of *S. europaea* was prepared as illustrated in section (2.4.1). A total of 1 mg/ml from each sample (total fatty alcohol, total hydrocarbon and total fatty acid methyl ester) was dissolved in 1 ml dichloromethane and analysed on GC/MS instrument (section 2.6).

Table 3.1 Compounds identified in alcohols fraction via GC-MS.

No	Compounds	Molecular formula	RT(min.)	Relative %
1	Heptacosane	$C_{27}H_{56}$	25.96	43.02
2	Octacosane	$C_{28}H_{57}$	27.26	45.62
			Total	88.64

Table 3.2 Compounds identified in hydrocarbon fraction of *S. europaea* via GC-MS.

No	Compounds	Molecular formula	RT(min.)	Relative %
1	β – Selinene	$C_{15}H_{24}$	13.37	76.09
2	Caryophyllene oxide	$C_{15}H_{24}O$	14.5	4.20
3	Pentadecanone	$C_{18}H_{36}O$	17.2	2.23
			Total	82.52

Table 3.3 Compounds identified in fatty acids ester fraction of *S. europaea* via GC-MS.

No	Fatty acids	Molecular formula	RT(min.)	Relative %
1	7-Hexadecanoic acid, methyl ester	$C_{17}H_{32}O_2$	13.3	2.23
2	Nonanedioic acid, dimethylester	$C_{11}H_{20}O_4$	13.94	1.72
3	Cyclopropane butanoic acid	$C_{25}H_{42}O$	16	0.43
4	Caffeine	$C_8H_{10}N_4O_2$	17.2	7.93
5	Isopropyl myristate	$C_{17}H_{34}O_2$	17.3	1.05
6	1,2,Benzenedicarboxylic acid,bis(2-methyl propyl)ester	$C_{16}H_{22}O_4$	17.5	1.98
7	9, 12, octadecatrienoic acid	$C_{27}H_{52}O_4$	18.049	17.47
8	Oleic acid, 3-(octadecyloxy) propyl ester	$C_{39}H_{76}O_3$	18.4	1.21
9	Heptadecanoic acid, 16 methyl -methylester	$C_{19}H_{38}O_2$	20	2.68
			Total	36.7

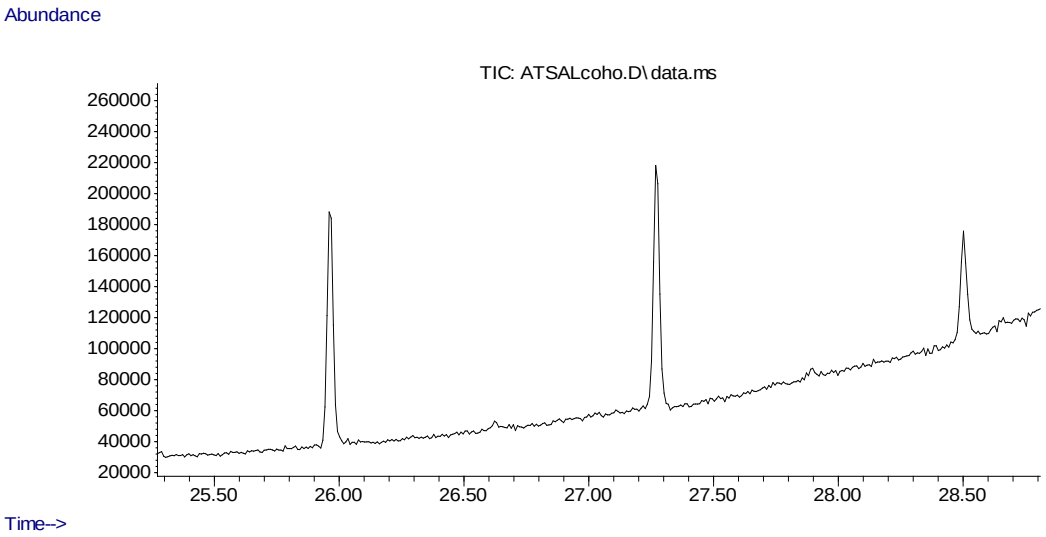


Figure 3.1 GC-MS analysis of alcohol fraction of *S. europaea*.

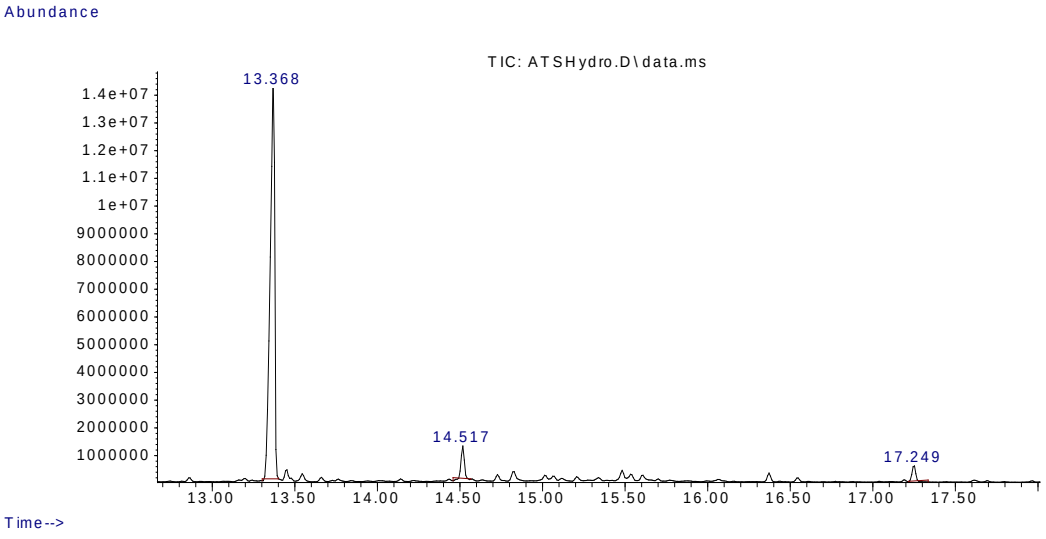


Figure 3.2 GC-MS analysis of hydrocarbon fraction of *S. europeae*.

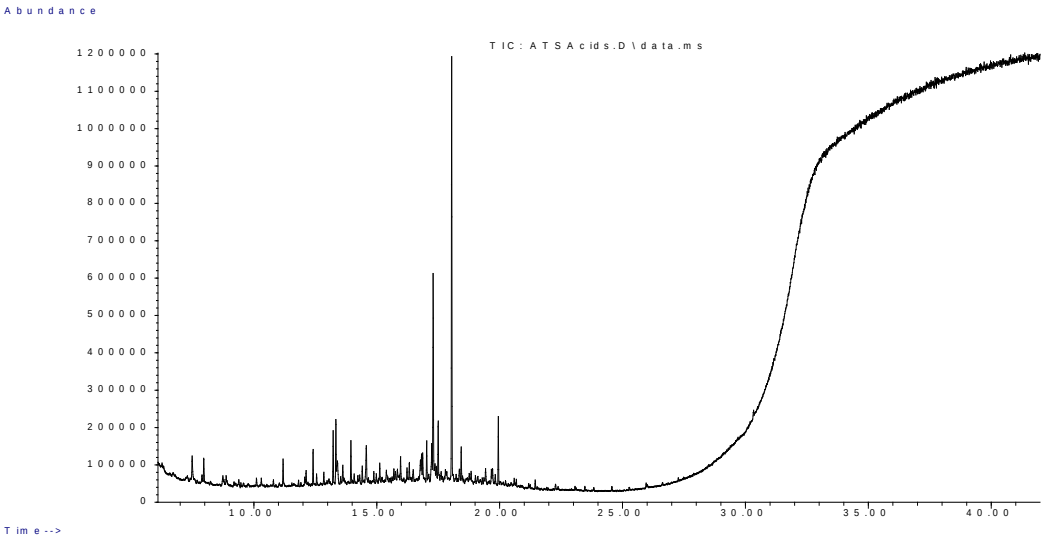


Figure 3.3 GC-MS analysis of fatty acid methyl ester fraction of *S. europaea*.

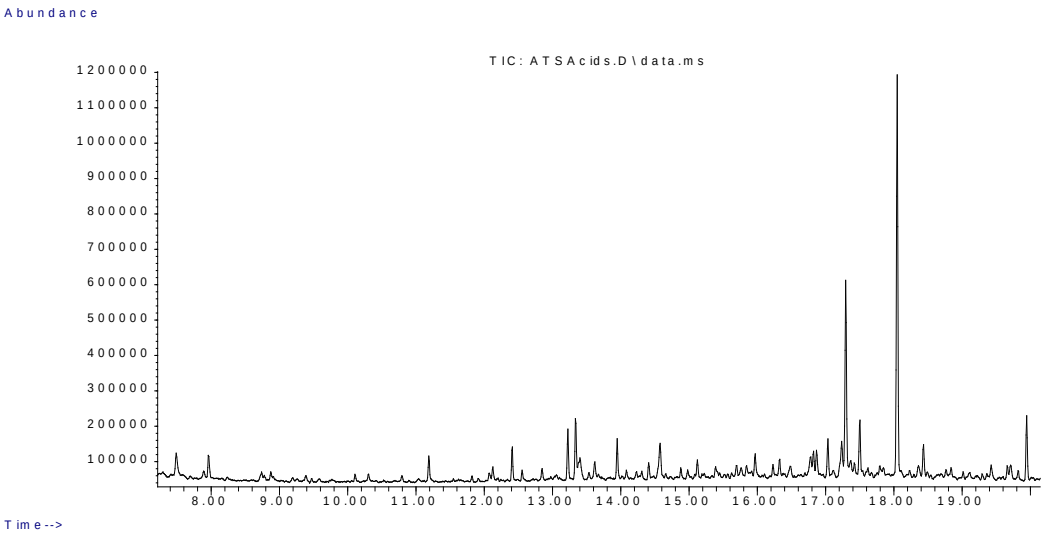


Figure 3.3 Continued.

Table 3.1 and Figure 3.1 show the compounds identified in the total fatty alcohols of *S. europaea*. Although the aim of the work was to identify the total fatty alcohols, the results also showed the presence of two hydrocarbons with high molecular weight. Table 3.2 and Figure 3.2 show the compounds identified in the hydrocarbons mixture of *S. europaea.* β - Selinene and caryophyllene oxide were also identified in the essential oil of *S. europaea* using GC/MS by (Pavlovic et al., 2006) as main constituents. The GC-MS analysis of total fatty acid methyl esters Table 3.3, Figure 3.3 showed the presence of 7-hexadecanoic acid, methyl ester (palmitic acid methyl ester) $C_{17}H_{32}O_2$. Palmitic acid ($C_{16}H_{32}O_2$) was isolated from the hexane extract of *S. europaea* (section 3.4.5), and this extract also showed the presence of **1** (bis (2-ethylhexyl) phthalate), thus providing additional confirmation of the presence of **1** in *S. europaea.* Another interesting peak present in the GC-MS of the Fatty acid esters of *S. europaea* was identified as caffeine (7.93%) on the basis of its mass and fragmentation pattern. This finding is surprising and it is possible that the caffeine detected was a contaminant and further work needs to be carried out in order to confirm or refute the presence of caffeine in *S. europaea*.

3.2 Preparation and yields of crude glycosides

The crude glycosides extract of *S. europaea* was prepared according to the method mentioned in section (2.4.1.2). Several batches of *S. europaea L.* herb were extracted using different amounts of herb and extraction conditions. The yields of crude glycosides obtained are shown in table (3.4).

3.3 Fractionation of crude glycosides of *S. europaea*

Fractionation of crude glycosides of *S. europaea* was carried out by using several adsorbent e.g. normal phase silica gel particle size (0.02-0.04 mm

- 0.04-0.06 mm), reverse phase silica gel C-18 (LiChropher ^{R} 100 RP-18E 12 µm), preparative TLC, gel filtration (Sephadex LH20 -sigma) and High performance liquid chromatography (HPLC).

3.3.1 Normal phase silica gel

200 mg of crude glycosides were chromatographed on 100 g silica gel for thin layer chromatography (section 2.5.1.) using a gradient system of $CHCl_3$:MeOH [(100:0), (98:2), (96:4), (94:6), (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90), (0:100), 200 ml each], 98 fractions 7 ml each were collected. Similar fractions were combined depending on TLC analysis results (silica gel 60 F_{254} aluminium sheets 20 × 20 cm plates) using chloroform: methanol: water (60: 40:10) as mobile phase and vanillin sulphuric acid as spray reagent), fraction A(10-12, 20 mg), B(13-37, 77 mg), C(38-50, 77 mg), D(60-99, 30 mg).

Table 3.4 Yields of crude glycosides obtained with different conditions.

Sample number	Plant material (g)	% of MeOH Solvent used	Amount of solvent (ml)	Extraction time (h.)	Amount of $CHCL_3$ (ml)	Amount of BuOH (ml)	Amount of MeOH (ml)	Amount of diethyl ether (ml)	Weight of crude glycoside (g)	The % of crude glycoside in the dried herb
1	75	80	700	23	15(3times)	15(3times)	10	15	0.81	0.607
2	75	80	1400	16	15(3times)	15(3times)	50	100	1.5	1.125
3	90	80	1400	24	15(4times)	15(6times)	55	60	1.61	1.449
4	90	80	1400	37	15(6times)	15(10times)	45	70	1.28	1.152
5	90	80	1500	16	15(6times)	15(6times)	55	40	1.20	1.08
6	90	80	1500	16	15(3times)	15(6times)	55	40	1.2	1.08
7	100	80	1400	23	15(6times)	15(12times)	55	170	1.28	1.28
8	90	80	1500	17	15(6times)	15(11times)	50	100	1.22	1.06
9	100	80	1500	24	15(6times)	15(6times)	50	100	0.90	0.70

Repeatedly, Fractions A, B, C, D, were further fractionated on a silica gel column eluted with $CHCl_3$:MeOH gradient, 36, 101, 68 and 56 fractions (12 ml) were collected from each column respectively. TLC analysis of the fractions was carried out. Although, the saponin mixture of crude glycosides was not separated, partial purification of nonpolar compounds in the mixture was achieved (purification of the main nonpolar compounds is described in (section 3.2.3). Table 3.5 shows the compositions of mobile phases which were used for analytical TLC (normal phase silica gel plates). A mobile phase of $CHCl_3$:MeOH:H2O, 60:40:10 was found to produce the best separation of crude glycosides constituents on analytical TLC plates.

Table 3.5 Mobile phases trialled (analytical TLC with silica gel plates).

Mobile phase	**% of solvents**
$CHCl_3$:MeOH:H_2O	40:60:10
$CHCl_3$:MeOH:H_2O	50:50:10
$CHCl_3$:MeOH:H_2O	60:40:10
$CHCl_3$:MeOH:H_2O	70:30:10
$CHCl_3$:MeOH:H_2O	70:30
$CHCl_3$:MeOH:H_2O	80:20
$CHCl_3$:MeOH:H_2O	90:10:5
$CHCl_3$:MeOH:H_2O	90:10:10
BuOH:CH3COOH:H_2O	50:10:40

Use of the normal phase method (section 2.5.1) to purify crude glycosides from an extract of *S. europaea* led to isolation of two nonpolar compounds, bis (2-ethylhexyl) phthalate (**1**), and palmitic acid (**2**). One disadvantage of this method is that some of saponin compounds were bound to the silica gel, which was recognized by TLC analysis to the crude glycosides extract

before do fractionation on the column chromatography and to the collected fractions from the column as few compounds were disappeared from the fractions.

3.3.2 Sephadex LH20

Another method applied to purify the crude glycosides extract was the use of Sephadex LH20 (section 2.5.4.). At this time 315 mg crude glycosides extract were applied to 100 g Sephadex LH20 (2.5 × 100 m), eluting with a $CHCl_3$:MeOH gradient. A total of 116 fractions were collected and TLC analysis was carried out for the fractions. Fractions were combined depending on their similarity on TLC plates. The column conditions are shown in the Table 3.6.

The fractions were combined as A1(2-23) 10 mg, B1(24-43) 20 mg, C1(44-48) 35mg, D1(49-63) 22mg, E1(64-95) 38mg, F1(96-100) 66mg, G1(101-112) 41mg, K1(113-116) 50mg. Fraction A(24-43) was a mixture of less polar compounds, whereas C1, D1, E1, F1, contained saponin compounds. Since the Sephadex column was shown to be effective in separating less polar compounds from the saponin fraction, this procedure was repeated several times in order to collect a large quantity of the saponins fraction for further purification using preparative TLC.

Table 3.6 Solvent conditions for Sephadex column fractionation of crude glycosides from *S. europaea.*

Mobile phase	% of compositions	Volume (ml)	Collected Fractions	Combined fractions weights not recorded)
$CHCl_3$	100	100	1	
$CHCl_3$:MeOH	90:10	100	2-6	
$CHCl_3$:MeOH	85:15	100	7-14	
$CHCl_3$:MeOH	70:30	100	15-23	A1(2-23)
$CHCl_3$:MeOH	60:40	100	24-31	B1(24-43)
$CHCl_3$:MeOH	50:50	100	32-42	C1(44-48)
$CHCl_3$:MeOH	40:60	100	43-53	D1(49-63)
$CHCl_3$:MeOH	30:70	100	54-60	C1(44-48)
$CHCl_3$:MeOH	20:80	100	61-69	D1(49-63)
$CHCl_3$:MeOH	10:90	100	70-83	
$CHCl_3$:MeOH	100	100	84-92	E1(64-95)
$CHCl_3$:MeOH	90:10	100	93-100	F1(96-100)
$CHCl_3$:MeOH	85:15	100	101-112	G1(101-112)
$CHCl_3$:MeOH	70:30	100	113	
$CHCl_3$:MeOH	60:40	100	114-115	K1(113-116)
$CHCl_3$:MeOH	50:50	100	116	

A total of 300 mg crude glycosides were fractionated on a 100 g Sephadex LH20 column (2.5 × 100 m), eluted with $CHCl_3$:MeOH (2L) gradient, 166 fractions 7 ml each collected. TLC analysis was carried out and fractions were combined depending on the similarity on TLC plates. Fractions were combined as fraction A2 (14-43, 30 mg), B2 (44-67, 76 mg), C2 (68-103, 35 mg), D2 (119-155, 95 mg). Fractions containing saponins were stored at 4^oC.

Several mobile phases were used as eluting solvent for crude glycosides fractionated on Sephadex LH20 column (5.5 × 58 cm), such as MeOH (100%), MeOH:$CHCl_3$ gradient system, MeOH:H_2O (50:50) and MeOH:H_2O:EtoAc (60:40:10). For the eluting solvent MeOH:H_2O (50:50), crude glycosides (100 mg) were dissolved in small quantity of MeOH:H2O (50:50) and fractionated on 70 g of Sephadex LH20. A total of 30 fractions of 14 ml each were collected, TLC analysis of the fractions was carried out and fractions were combined depending on the similarity on TLC plates.

A total of 300 mg crude glycosides were fractionated on Sephadex LH20 column (5.5 × 58 cm), MeOH:H_2O:EtoAc, 60:40:10, 88 fractions 7 ml each were collected. TLC analysis was carried out for the fractions and fractions were combined depending on the similarity on TLC plates. Elution with MeOH:H_2O:EtoAc, 60:40:10, was repeated two times more with the same conditions in order to collect a large quantity of saponins fraction.

The gel filtration (Sephadex LH-20) method to separate the saponin compounds from crude glycosides of *S. europaea* was successful in the separation of nonpolar compounds from the saponins fraction, but the saponins compounds were not separated. The difficulties in separation of saponins might be because they have similar molecular weight or they are isomers for each other. The saponin fraction was further purified using preparative TLC and reverse phase silica gel column.

3.3.3 Preparative Thin Layer Chromatography (PTLC)

Fraction D2 (119-166, 95 mg), portion of crude glycosides eluted with $CHCl_3$:MeOH (85:15, 80:20, 70:30, 60:40, (section 3.2.1.2), was subjected to preparative TLC (silica gel 60 F_{254} aluminium sheets 20×20 cm plates) using chloroform:methanol:water, 60:40:10 as a mobile phase and vanillin sulphuric acid as spray reagent), 17 plates were used, 5 mg of fraction D2 was applied on each plates, the sample was firstly dissolved in a small quantity of methanol, and a capillary tube (size 5µm) was used to apply the sample on silica gel plates. A total of four applications (5 mg)

were applied to each plate, the TLC plates were allowed to dry completely, then placed in chromatographic tank containing $CHCl_3$:MeOH:H_2O, 60:40:10, after the solvent developed through the plate.

TLC plates were removed from the tank, dried completely then placed again in a fresh solvent. The process was repeated three times. The TLC plates were dried and checked under UV, the bands marked by pencil then removed by small spatula. The combined bands were collected in small vials and extracted with methanol, sonicated for 15 minutes and filtered. The methanol was evaporated using rotary evaporator. Preparative TLC process was successful in separation one of saponin compounds (1 mg), identification of the compound was also achieved (saniculaside N).

The use of preparative TLC to purify the saponins fraction (resulting from fractionation of crude glycoside on Sephadex LH-20 column) was effective (Saniculoside N) (1 mg), the quantity was enough to do ^{1}H NMR and mass analysis. At this time the identification of saponin compound was achieved by comparison the ^{1}H NMR data of the saponin compound with the literature data. One disadvantages of preparative TLC method is that 17 plates were used to analysis (85 mg) of saponins fraction, but only (1 mg) of pure compound was yielded. The preparative TLC method is consuming both TLC plates and saponin fraction. Thus, another method to purify the saponin fraction with higher yield was required. Four saponin compounds are shown on the TLC plate.

3.3.4 Reverse phase silica gel C-18

Crude glycosides fractions C1 + D1 fractionated on a 35 g reverse phase silica column (LiChrospher ^{R} 100 RP-18E 12 µm) eluting via H_2O:MeOH gradient starting from 100:0, to 0:100. A total of 67 fractions of 7 ml each were collected and the fractions were combined depending on the similarity on TLC plates. TLC was achieved using silica gel 60 F_{254} aluminium sheets 20×20 cm plates, using $CHCl_3$:MeOH:H_2O (60: 40:10)

as mobile phase and vanillin sulphuric acid as spray reagent. A total of 15 main fractions were obtained [A(2-4), B(5-10), C(11-12), D(13-15), E(16-22), F(23-24), G(25-30), K(33-38), L(39-43), M(44-51), N(54-57).

Fractions C(11-12), D(13-15), E(16-22) were (20 mg total) were combined and further fractionated on a 30 g reversed phase silica gel C_{18} column (5.5 × 58 cm) using $CHCl_3$:MeOH mixture as eluting solvent. This process afforded 35 fractions of 7 ml each. Fractions numbered 13, 14, 15 each contained mainly one compound, but the quantity was very small 0.3 mg.

This procedure was repeated after collection fractions F(23-24), G(25-30), but the eluting solvent was changed to $CHCl_3$:MeOH:H_2O gradient, 83 fractions 21 ml each were collected, TLC carried out. Table 3.7 the compositions of mobile phases which tested for revers phase silica gel plates, The mobile phase of MeOH:H_2O (85:15) was found produce the best separation of crude glycosides constituents on the analytical TLC plates.

Table 3.7 Compositions of mobile phases tested for the use with revers phase silica gel plates.

Mobile phase	% of solvents
MeOH:H_2O	100:0
MeOH:H_2O	90:10
MeOH:H_2O	85:15
MeOH:H_2O	80:20
MeOH:H_2O	75:20
MeOH:H_2O	60:40
MeOH:H_2O	50:50
MeOH:H_2O:$CHCl_3$	50:50:1
MeOH:H_2O:$CHCl_3$	60:40:1
MeOH:H_2O:$CHCl_3$	70:30:1
MeOH:H_2O:DCM	50:50:1
MeOH:H_2O:DCM	60:40:1
MeOH:H_2O:DCM	70:30:1
MeOH:H_2O:DEE	50:50:1
MeOH:H_2O:DEE	60:40:1
MeOH:H_2O:DEE	70:30:1
MeOH:H_2O:Hex	50:50:1
MeOH:H_2O:Hex	60:40:1
MeOH:H_2O:Hex	70:30:1
MeOH : H_2O:CH_3COOH	60:40:10
MeOH:H_2O:$(CH_3)_2CO$	60:40:10
MeOH:H_2O:CH_3CN	60:40:10
MeOH:H_2O:BuOH	60:40:10
MeOH:H_2O:Toluene	50:50:1
MeOH:H_2O:Toluene	60:40:1
MeOH:H_2O:Toluene	70:30:1
MeOH:H_2O:EtOH	60:40:10
MeOH:H_2O:EtOAC	70:30:1
MeOH:H_2O:EtOAC	70:30:2
MeOH:H_2O:EtOAC	60:40:2
MeOH:H_2O:EtOAC	60:40:3
MeOH:H_2O:EtOAC	50:50:2

DCM : Dichloromethane.

DEE : Diethyl ether.

Hex : Hexane.

EtOH : Ethanol.

EtOAC : Ethyl acetate.

The disadvantage of using reverse phase silica gel (particle size 0.02-0.04 mm, 0.04-0.06 mm) to purify either the crude glycosides extract or saponins mixture was the yield of resulting pure compounds. One reason for that is that the saponin compounds occur in each sub fraction, in addition to by repeating the fractionation method four or three times we end up with small quantity of pure compounds.

Use normal phase silica gel, Sephadax LH20, Preparative TLC and reverse phase silica gel to do fractionation of crude glycosides of *S. europaea* resulted in isolation of 5 compounds and partially purified saponins fractions. Compound **1** was identified as (Bis-2-ethyl hexyl phthalate), palmitic acid **(2)**, rosmarinic acid **(3),** saniculoside N **(4)** and compound **(5)** was composed from three sugars named sucrose, glucose and fructose. Partially purified saponins fractions were combined depending on the similarity on TLC into three main fractions A (218.07 mg), B (363.37 mg) and C (89 mg), to further purify by high performance liquid chromatophraphy (HPLC) techniques.

3.4 Natural products isolated from *S. europaea*

The following section is about the isolation and structure elucidation of the compounds isolated from the 80% methanolic extract of *S. europaea*.

3.4.1 Isolation of bis (2-ethylhexyl) phthalate 1

A total of 1 g of crude glycoside extract was dissolved in methanol 10 ml and mixed with 20 mg of silica gel (section 2.5.1). The methanol was evaporated in *vacuo* at 45 ^{o}C until dryness to give a homogenous powdered extract. This powder was transferred to a silica gel column (30 g

of silica in 200 ml of chloroform). Fractions of 20 ml each were collected. Fractions were monitored using TLC with $CHCl_3$:MeOH:H_2O (60:40:10) as a developing solvent (section 2.5.5). The data are summarized in the Table 3.8.

Table 3.8 Conditions of column chromatography of isolation of 1.

Mobile phase	% of compositions	Volume (ml)	Fraction number
$CHCl_3$	100	100	1-5
$CHCl_3$:MeOH	98:2	100	6-15
$CHCl_3$:MeOH	95:5	100	16-20
$CHCl_3$:MeOH	90:10	100	21-24 (compound **1)**
$CHCl_3$:MeOH	85:15	100	25-30
$CHCl_3$:MeOH	70:30	100	31-37(saponin fraction)
$CHCl_3$:MeOH	60:40	100	38-41
$CHCl_3$:MeOH	50:50	100	42-45
$CHCl_3$:MeOH	40:60	100	46-49
$CHCl_3$:MeOH	30:70	100	50-54
$CHCl_3$:MeOH	20:80	100	55-59
$CHCl_3$:MeOH	10:90	100	60-64
MeOH	100	100	65

3.4.1.1 Further purification of 1

Fractions (21-24) Table 3.8 eluted with $CHCl_3$:MeOH (90:10) were found to contain only one compound with main impurity, so they were combined, concentrated to a small volume and further purified using a Sephadex column (25 g) in 150 ml methanol. The data were summarized in table 3.6. The fractions 6-10 were found to contain one compound (6 mg). The TLC of **1** is Figure 3.4.

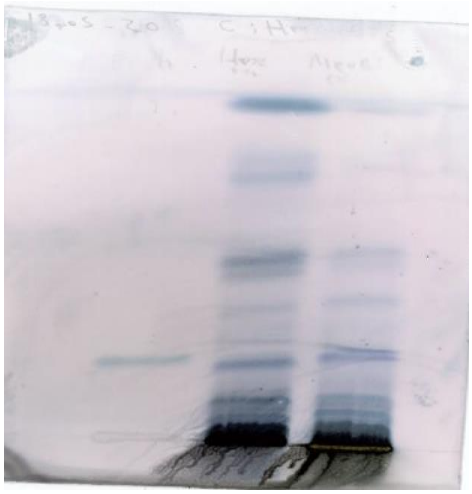


Figure 3.4 TLC analysis of 1

Figure 3.4 is shown TLC plate (silica gel 60 F_{254} aluminium sheets 20×20 cm, chloroform:methanol:water, 60:40:10, vanillin sulphuric acid) of **1** the bands from the left to the right represent bis (2-ethylhexyl) phthalate, hexane fraction from methanol extract of *S. europaea* and methanol extract of *S. europaea*.

Table 3.9 Conditions of sephadex column of further purification of 1.

Mobile phase	**% of compositions**	**Volume (ml)**	**Fractions (10 ml)**
$CHCl_3$	100	50	1-5
$CHCl_3$: MeOH	90:10	50	6-10 compound **1**
$CHCl_3$: MeOH	85:15	50	11-15
$CHCl_3$: MeOH	70:30	50	16-20
$CHCl_3$: MeOH	60:40	50	21-25
$CHCl_3$: MeOH	50:50	50	26-30
$CHCl_3$: MeOH	30:70	50	31-35

3.4.1.2 Structure elucidation of 1

Compound **1** was isolated as a yellow oil from the crude glycosides extract of *S. europaea.* TLC analysis of **1** on silica gel 60 F_{254} showed quenching at 254 nm but did not fluoresce at 375 nm and showed a dark blue spot after vanillin / sulphuric acid spraying (Figure 3.4). The ES+ mass spectrum of **1** Figure 3.5 showed a molecular ion peak $[M+H]^+$ at m/z = 391.2 suggesting a molecular weight of 390; other peaks were observed at m/z = 413.2 $(M + Na)^+$, 804.5 $(2M + Na)^+$.

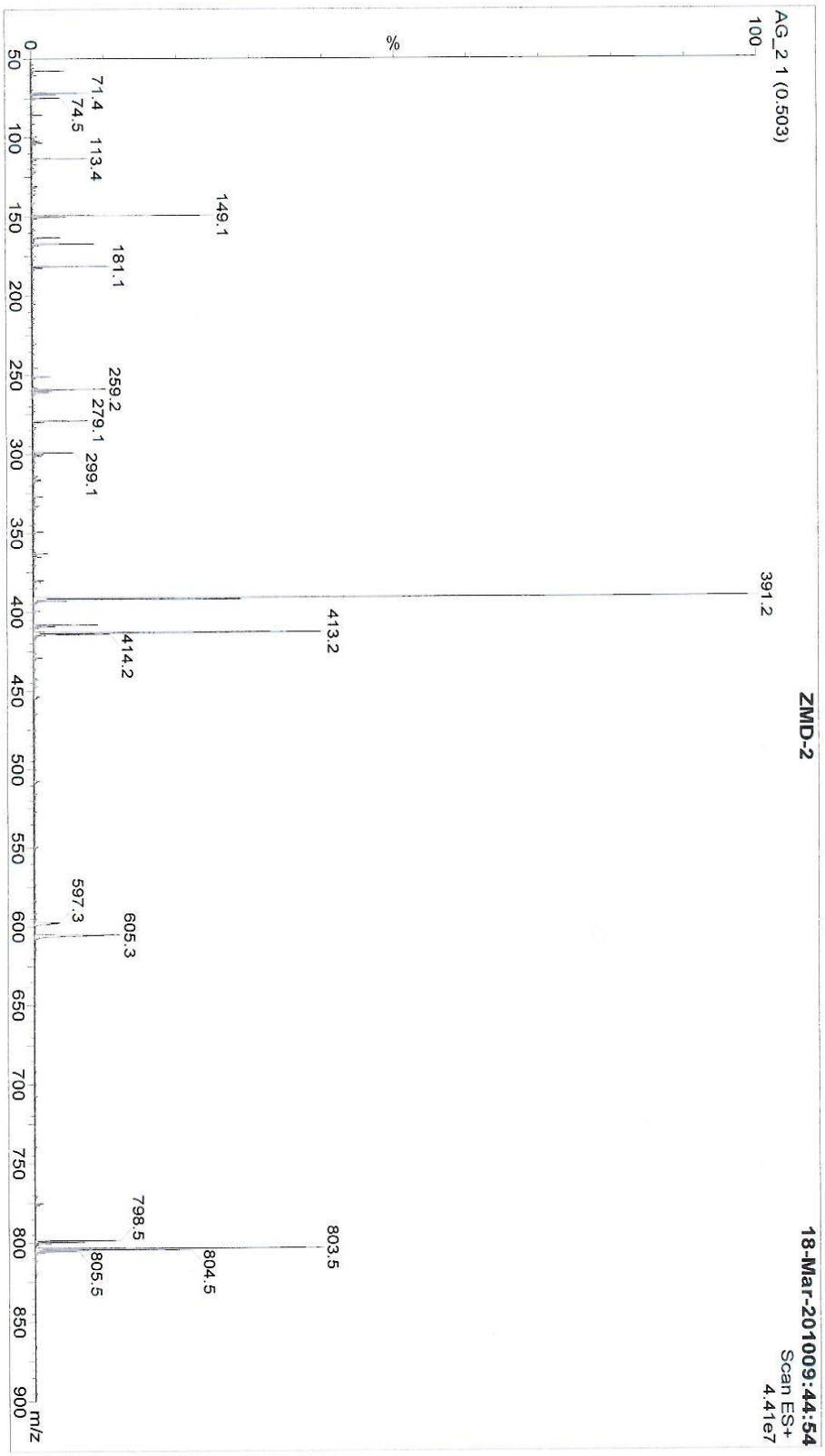


Figure 3.5 ES + mass spectrum of 1.

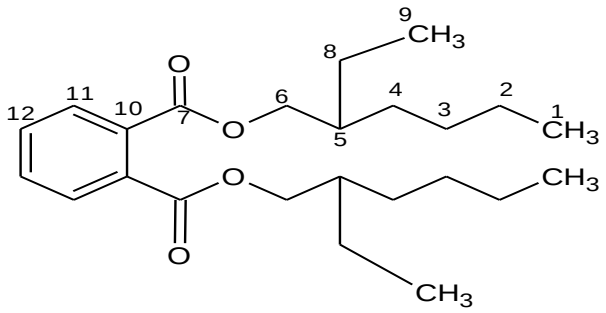


Figure 3.6 Bis (2-ethylhexyl) phthalate 1.

The ^{1}H NMR and ^{13}C NMR data is shown in Table 3.10. The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **1** Figure 3.7 showed several signals in the up field region between δ 0.7 and δ 1.9 which are characteristic for CH_2, CH_3 protons and signals in the downfield region between δ 7.6 and 7.9 which are characteristic for aromatic protons. The signals at δ 0.8 and 0.89 are triplet indicates the presence of two methyl groups. The 6H signal at δ 1.27 suggest the present of 5 ($-CH_2-$) and 1H signal at δ 1.65 indicates the presence of a methine CH. The down field shift suggests that this group is attached to an oxygen atom. The peaks in the downfield region of the ^{1}H NMR spectrum showed the presence of two aromatic protons at δ 7.50 (1H, dt, *J* = 2.2, 6.3 Hz, H-12), 7.65 (1H, dd, *J* = 2.2, 6.3 Hz, H-11).

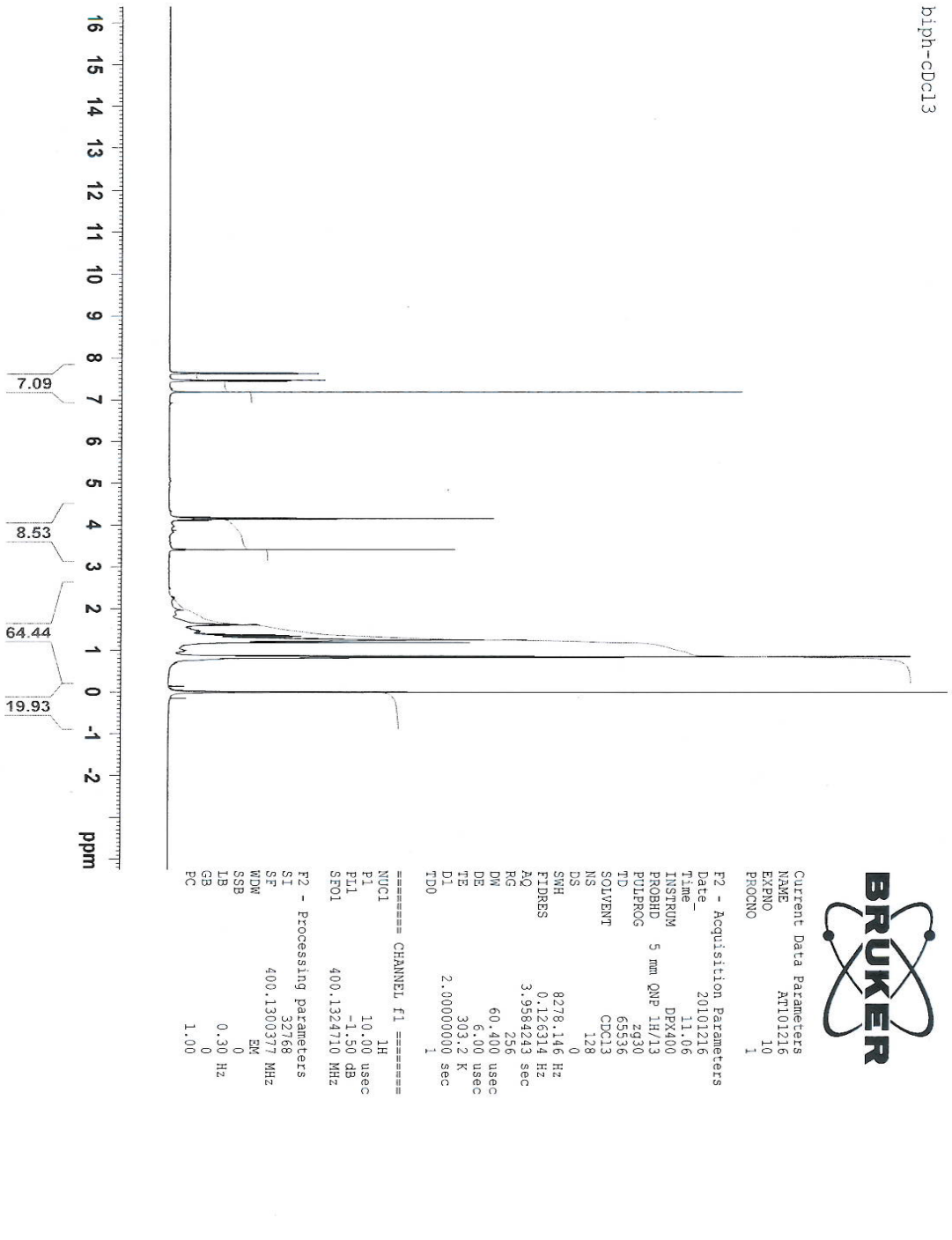


Figure 3.7 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 1.

The ^{13}C NMR (400 MHz, $CDCl_3$) spectrum Figure 3.9 showed 12 signals. Eight signals were observed in the up field region with chemical shifts between δ 11 and 68 ppm; the DEPT spectrum of **1** Figure 3.10 indicated the presence of two methyl groups, four methylene groups and one for a methine group. The peak at δ 68 is characteristic for a carbon atom bonded to an oxygen atom. In the downfield region of the ^{13}C NMR spectrum three aromatic carbons (two methine, one quaternary) and one carbonyl carbon were observed.

From the above data a total of 12 carbon signals and signals for 19 protons are observed. In addition, there is an evidence for two oxygen atoms, one carbonyl and one other oxygen. This gives an empirical formula for **1** is $C_{12}H_{19}O_2$, The mass of this formula is 195 (12C = 144, 19H = 19, 2O = 32). The mass spectrum showed that the molecular weight is actually 390; therefore the molecular formula of **1** is $C_{24}H_{38}O_4$. The six aromatic carbons suggest that the molecule contains one six member atoms ring with four adjacent protons Figure 3.8. Therefore, there are two side chains, each one contains two methyl groups four methylene groups, one methine group attached to oxygen atom and carbonyl group. Moreover, the side chain most be branched on account of two methyl groups.

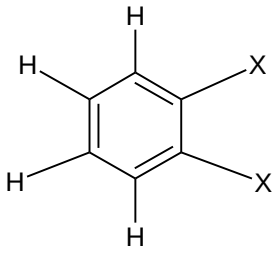


Figure 3.8 Partial structure of 1.

Figure 3.9 ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of 1.

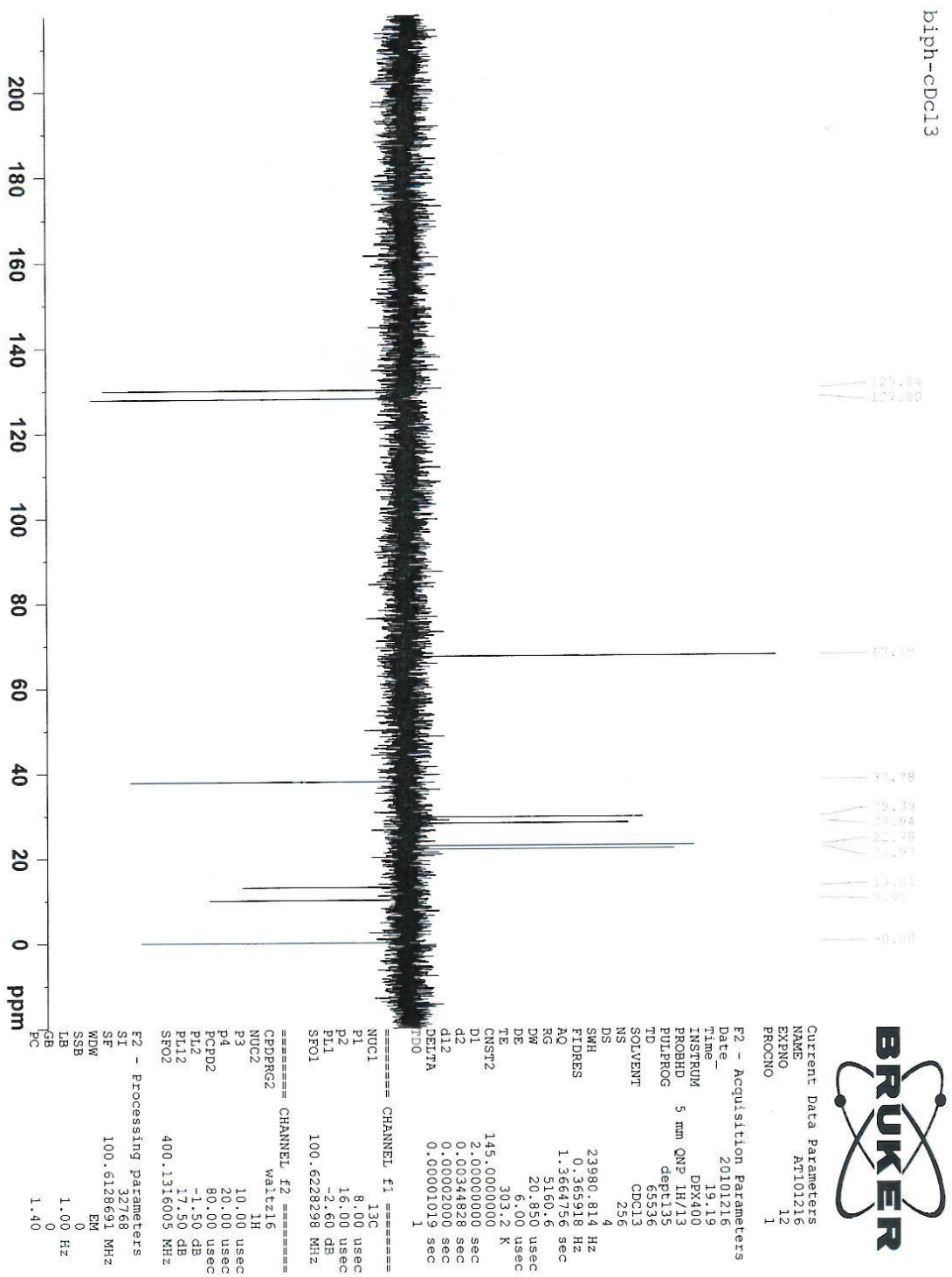


Figure 3.10 DEPT spectrum (400 MHz, $CDCl_3$) of 1.

Table 3.10 ^{13}C and ^{1}H NMR chemical shift and DEPT data (400 MHz, $CDCl_3$) of 1.

Carbon number	^{13}C NMR, δ, PPM	DEPT	^{1}H NMR, δ, PPM
1	14	CH_3	0.89 (3H, t, H-1)
2	29	CH_2	1.27 (2H, m, H-2)
3	23	CH_2	1.27 (2H, m, H-3)
4	30	CH_2	1.27 (2H, m, H-4)
5	39	CH	1.65 (1H, m, H-5)
6	68	CH_2	4.12 (2H, m, H-6)
7	167	O-C=O	-
8	24	CH_2	1.27 (2H, m, H-8)
9	11	CH_3	0.80 (3H, t, H-9)
10	133	C	-
11	129	Aromatic CH	7.65 (1H, dd, *J* = 2.2, 6.3 Hz, H-11)
12	131	Aromatic CH	7.50 (1H, dt, *J* = 2.2, 6.3 Hz, H-12)

All the above data are in accordance reported spectral data of bis (2-ethylhexyl) phthalate (**1**) (Kui, 2009). Therefore, it can be concluded that **1** is bis (2-ethyl hexyl) phthalate (**1**) Figure 3.6.

Bis (2-ethylhexyl)phthalate is well known plasticizer and it is used in manufacturing of many in industrial products such as plastic polymers, pharmaceuticals, cosmetics, inks and adhesives. Although, **1** has low acute toxicity it is classified as a probable human carcinogen and is expected to be a human endocrine disruptor. In animal studies using oral dosing for short periods, **1** is well known for its reproductive toxicity, especially for its testicular toxicity (Nelson et al., 2013) .

3.4.2 Occurrence of 1 in nature

3.4.2.1 Occurrence of 1 in foods

Presence of **1** in food (Stilton cheese) was reported by Michael *et al* (2013). In their study they were measured the abundance of radiocarbon in 5 samples of **1** isolated from Stilton cheese by accelerator mass spectrometry (AMS) to calculate the carbon fraction created from contemporary biogenic bases. The aim of this study was to confirm whether it's presence in food as a contaminate from industrially synthesized **1** or a naturally inherent compound. Five ≈ 90 µg quantities of **1** were isolated from 12 Kg of Stilton cheese using silica gel, size exclusion and HPLC. Samples masses were measured via GC-MS analyses prior to combustion and manometry. The mean ^{14}C-corrected contemporary carbon fraction of **1** in the isolated was 0.235 ± 0.073 (1σ and ± 0.091 at the 95% confidence level). It was concluded that the high percentage of **1** in Stilton cheese results from anthropogenic sources, although with a significant naturally occurring component (Nelson et al., 2013).

3.4.2.2 Occurrence of 1 in water treatment plants

A type of fungus named Actinomycete was isolated from the leaf litter sample from streams. The bioactive components were purified by silica gel column chromatography and identified by Gas Chromatography-Mass Spectrometry. Ten compounds were identified between them bis (2-ethylhexyl) phthalate **1** (33.19%).

The plasticizers and their degeneration in streams of a large urban sewage treatment plant were studied by (Johnson et al., 2012). (Barnabe *et al* 2008) The results revealed that **1** is present at high concentrations in the process streams, influents, treated effluent and solid residues of the treatment plant in Montreal, Canada. Degradation of **1** lead to the

formation of three compounds, 2-ethylhexanal, 2-ethylhexanol and 2 ethylhexanoic acid. These compounds were earlier reported in laboratory work. **1** and it's three degradation compounds were found in the influent and solids that were analysed. The results suggest that the treatment plant does not completely remove plasticizer from the influent and may be considered to be as an important source of these compounds and their degradation products in the environment (Barnabé *et al*., 2008).

3.4.2.3 Occurrence of 1 in marine organisms

Identification of bioactive components of marine *sediment* by gas chromatography was evaluated by Sudha Srikesavan and Masilamani (2012). **1** was found to be one of the marine constituent at percentage (14.13%), which also reported to have antibacterial activity (SudhaSrikesavan and Selvam, 2012).

3.4.2.4 Occurrence of 1 in bacteria organisms

One of bioactive constituents produced by *Nocardia levis* MK-VL_113 & *Streptomyces tendae* TK-VL_333 was **1**. The cytotoxicity activity of the compound was also evaluated IC_{50} values of 86.21 µg/ml, 119.89 µg/ml on cell lines U-937 and HL-60 respectively (Alapati and Muvva, 2013).

3.4.3 Pharmacological actions of 1

Habib and karim (2009) isolated **1** from the flower of *Calotropis gigantean*. They reported that **1** has been reported to be present in *Alchornea cordifolia* (Mavar *et al*., 2008), *Aloe vera* (Lee K. F. *et al*., 2000), *Euphorbia cyparissias* and *Euphorbia seguieriana* (Tothsoma *et al*., 1993). Habib and karim were also studied the antimicrobial and cytotoxic activity of **1** (Habib and Karim, 2009).

The shed dried plant material of *Calotropis gigantean* (1.0 kg) was extracted with ethyl acetate (1.5 L) at room temperature to yield (38 g) crude ethyl acetate extract of which 10 g was fractionated on normal phase silica gel for column chromatography and preparative to yield (98 mg) of **1** (Habib and Karim, 2009).

The minimum inhibitory concentrations of **1** (MIC_S) against bacterial species, *Staphyloccus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Sarcina lutea*, *Escherchia coli*, *Shigella sonnei*, *Shigella shiga* and *Shigella dysenteriae* was 64, 32, (no inhibitory), 32, 64, 64, 64 and 128 respectively.

The antifungal activity of **1** was carried out against four fungia species. The results revealed that **1** has activity against one species (*Aspergillus flavus*), the inhibition zone at doses 100 and 200 µg/disc was 08 and 11 respectively. Moreover, **1** showed high toxicity against brine shrimp nauplii (LC_{50} 9.19 µg/ml).

Habib and karim (2009) stated that the presence of **1** in flowers of *Calotropis gigantean*, was not as a contaminant from solvents, was further confirmed by GC-MS analysis (first confirmation was isolation of **1** from flowers of *Calotropis gigantean*, by using silica gel chromatography as mentioned above). The plant flowers were not preserved in plastic bags, so these could be discounted as a source of **1**. The authors reported that their study could not determine if **1** is synthesized by the plant, absorbed by the roots or from the external atmosphere (Habib and Karim, 2009).

Compound **1** was also isolated from *Ricinus communis Linn*. (*Euphorbiaceae*) (Pateh, 2009), Nuclea officinalis leaves (Kui, 2009), culture broth of a fungus strain No. 36 *Pencillium olsonii* (Amade *et al*., 1994).

Lee *et al.* (2000) examined the antitumor effect of **1** which was isolated from *Aloe vera* in human and animal cell lines. **1** was tested at three concentrations (100, 10 and 1) µg/ml against three leukaemic cell lines K562, HL60 and U937. **1** showed an inhibitory activity of 95, 97 and 95 % in K562, HL60 and U937 respectively at concentration of 100 µg/ml. At 10 µg/ml of **1,** the cells lines K562, HL 60 and U 937 showed growth inhibition of 74, 83 and 81 %. While at 1 µg/ml of **1** exhibited an inhibitory activity of 50, 51 and 52 % in K562, HL 60 and U 937 (Lee *et al*., 2000).

Zanotelli, *et al* (2009) investigated the effect of **1** on growth of larva and adult of guppy fish less than one week old at the start of the experiment at concentration present in nature and under conditions that might emphases harmful concerns. Two concentrations of **1** (0.1 and 10 µg/L) were applied constantly for 91 days. After 14 days of beginning of experiment the length of guppy fish was measured. The results revealed that the guppy fish treated with 10 µg/L of **1** undergo significant decrease in body length compared with control animals. The body weight was also reduced but this was after 91 days of treatment. The percentage decrease in body length was 15% and 40% for concentrations of **1** and 10 µg/L respectively, while the percentage of reduction in weight was 40% and 70 % for the both concentration respectively. **1** is widespread used plastic softener; as a result it is present in large number of plastic goods. It has the ability to transfer into the environment over time leading to universal contamination (Zanotelli *et al*., 2010).

Studies on skin penetration and metabolism of **1** was carried out by Hopf *et al*. **1** is assumed to cause endocrine disruption; it might have low dermal absorption. The aim of the study was to determine the permeation parameters of **1** and evaluate of skin **1** metabolism in individuals who highly unprotected to this lipophilic, low volatile material. Skin removed by surgery from patients who undergoing abdominoplasty was directly dermatomed (800 µm) and mounted on flow- through diffusion cells. The cells were injected into the skin in two forms pure **1** and emulsified in

aqueous solution (166 µg/ml). **1** transformed human fessible skin only in form of metabolite MEHP (mono-(2-ethyl-5-hydroxyhexyl phathalate). After 8 h of exposer, **1** permeated through human skin as MEHP only when emulsified in aqueous solution (Hopf *et al*., 2014).

3.4.4 Determination the origin of 1 in *S. europaea*

HPLC was used in order to quantify the amount of **1** present in different extracts of *S. europaea.* Method for HPLC analysis of **1** was as for the methods used for HPLC analysis of crude glycosides of extract of *S. europaea* (section 2.4.1.2). In order to determine the retention time of **1** in the HPLC chromatogram two samples (1 mg/ml and 0.1 mg/ml of **1** are dissolved in methanol (HPLC grade) and injected to a reverse phase HPLC column (10 × 225 mm, loop 100 µl) eluting with CH_3CN:H_2O:isopropanol, 35:60:5, flow rate **1** ml/min. This mobile phase gave a broad peak, therefore, the mobile phase was changed to $CHCl_3$:MeOH:H2O (60:45:5) which gave a sharp peak. Several concentrations of **1** were run as illustrated in the Table 3.11 is shown concentration of **1** vis area of the peak. Figure 3.12 is showing HPLC analysis of a standard sample of 1 (0.5 mg/ml).

Table 3.11 Analysis of standard sample of 1 (mg/ml) via HPLC

Conc. of 1 (mg/ml)	**Peak area**			**Average**	**SD**
0.125	9.005	9.532	9.63	9.38	0.33
0.2	20.033	19.015	20.47	19.84	0.74
0.25	25.04	21.496	24.98	23.83	2.03
0.3	28.6	30.446	30.85	29.96	1.20

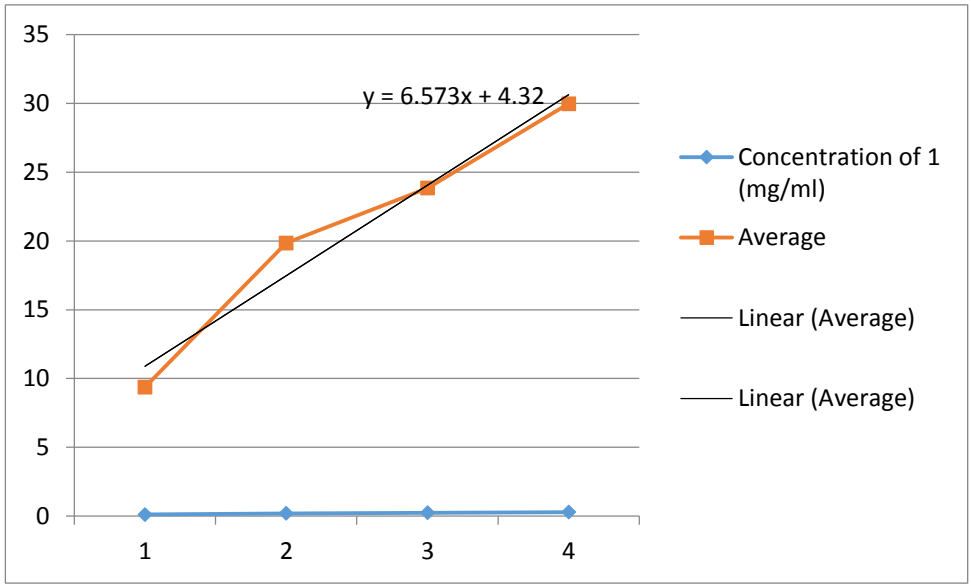


Figure 3.11 Graph showing a relationship between conc. of 1 and peak area.

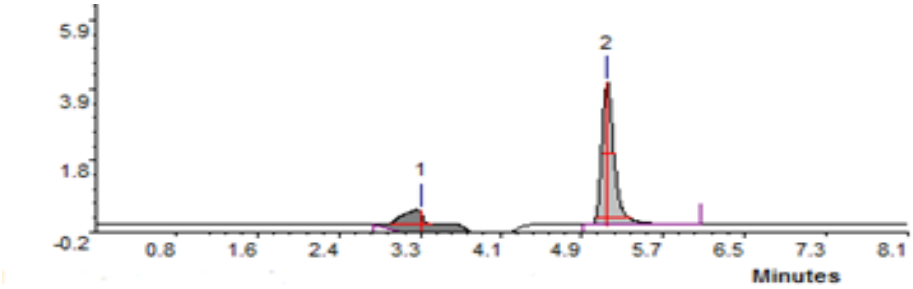


Figure 3.12 HPLC analysis of a standard sample of 1 (0.5 mg/ml), peak 2 indicates 1.

3.4.4.1 Analysis of 1 in total methanol extract of *S. europaea*

To confirm the presence of **1** in the total methanol extract of *S. europaea* (5 g) of the plant was extracted three times with 80 % methanol (3× 50 ml) at room temperature. The methanol extract was concentrated using rotary evaporator (yield 89.3 mg). The concentration of **1** in the methanol extract

is expected to be higher than that in the crude glycosides extract, since the latter was prepared from the methanol extract after several steps of partition with organic solvents such as hexane, chloroform, ethyl acetate, butanol (The crude glycosides extract was prepared from the butanol extract, therefore it is expected that **1** is extracted within each solvent as it is non-polar compound.

A concentration of 0.5mg/ml of methanol extract was analysed by HPLC. Using reverse phase silica gel column (10 × 225 mm, flow rate 2 ml/min) eluted with $CHCl_3$:MeOH:H_2O, 60:45:5, the concentration and the area of the peak of **1** on the chromatogram was recorded as illustrated in the Table 3.12. Figure 3.13 chromatogram is showing HPLC analysis of *S. europaea* methanol extract (0.5 mg/ml). Figure 3.14 is shown HPLC analysis of *S. europaea* methanol extract (0. 5 mg/ml) plus 0.2ml of **1** (1 mg/ml) injection loop 20 µl.

Table 3.12 Analysis of *S. europaea* methanol extract for compound 1.

Conc. of MeOH extract mg/ml	Area of the peak at RT = 5 min.			Average	SD
0.5	18.06	33	20	23.68	8.12

For *S. europaea* methanol extract, area of the peak of 0.5 mg/ml value fill in the area of the peak range of the standard curve Figure 3.8, therefore this value can be used to calculate the concentration of **1** in methanol extract. Rearrange SC equation.

X=(y+0.0396)/1.1029

X=(23.69+0.396)/1.1029

X= 21.5

Factor in for weight of solid per volume of solvent; methanol extract was prepared as 15g of material in 150 ml of 80 % methanol. It had a concentration of 100 mg/ml. Hence 21 mg of **1**.

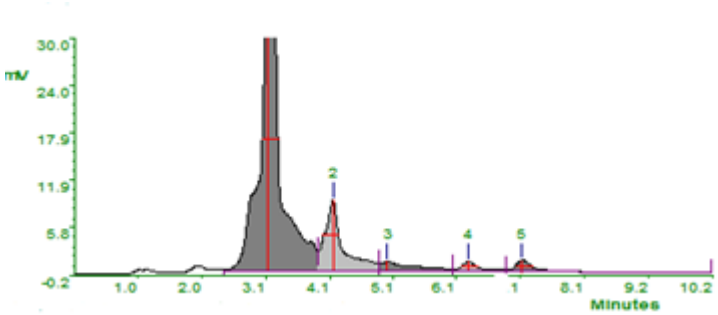


Figure 3.13 HPLC analysis of *S. europaea* methanol extract (0.5 mg/ml).

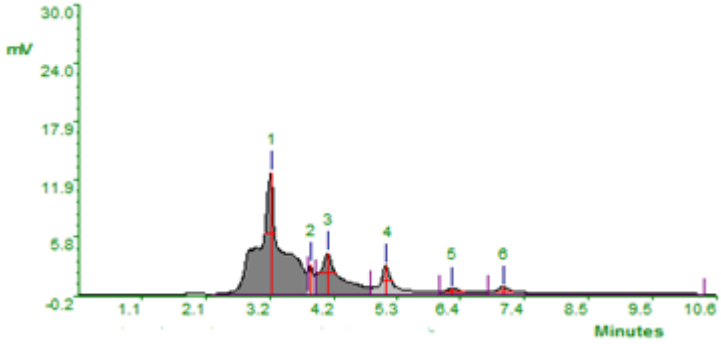


Figure 3.14 HPLC analysis of *S. europaea* methanol extract (0. 5 mg/ml) plus 0.2 mg/ml of 1.

3.4.4.2 Analysis of 1 in hexane fraction of S. europaea extract

A total of 100 g of *S. europaea* was macerated with (500 ml) of 80% methanol at room temperature for 5 days. The methanolic extract was concentrated in *vacuo* at 45 C° till dryness to yield (5.49 g), dissolved in (50 ml) distilled water. The aqueous phase was extracted with hexane (3 × 300 ml), followed by chloroform (3 × 300 ml), ethyl acetate (3 × 300 ml)

and butanol (3 × 50 ml). Anhydrous sodium sulphate was added to the solvents.

The solvents were filtered and concentrated *in vacuo* at 50 C°, to yield (15.73 mg), (76.74 mg), (1.16 g) and (3 g) of hexane, chloroform, ethyl acetate and butanol extracts respectively. The butanol extract was dissolved in (50 ml) of methanol and added to (200 ml) of diethyl ether to yield a white precipitate of crude glycosides extract (1.8g).

TLC analysis was carried out for the four extracts, in addition to standard sample of bis (2-ethylhexyl) phthalate **1**; the results showed that **1** is present mainly in the hexane extract, although, a small amount has extracted with the chloroform extract.

(2.5 L) of analytical grade hexane solvent was evaporated to dryness using a rotary evaporator. Analytical TLC was carried out for the residue to find out whether the **1** is contamination from the solvent or it present mainly in the plant. The TLC result showed that the residue did not contain any compound; this indicates that **1** is present in the plant and is not contaminant from the solvents.

In order to determine the concentration of **1** in the hexane extract, 2 ml (1mg/ml) of hexane extract was run on HPLC reverse phase silica gel (C_{18}) column (150 × 10 mm, 100 µL loop, flow rate 2 ml/min.) eluted with $CHCl_3$:MeOH:H_2O (60:45:5). The HPLC chromatogram showed a small peak at retention time of (5.1 min.) which is assigned to **1**, this process was repeated three times in order to show the reproducibility, retention time, area and the concentration were recorded each time, the area average was calculated. When three drops of standard **1** added to the hexane extract, the peak at retention time (5.2 min.) become sharper. Furthermore, a sample of standard **1** (0.5 mg/ml) was run on HPLC column reverse phase silica gel (C_{18}) column (150 × 10 mm, 100 µL loop, flow rate 2 ml/min.) eluted with $CHCl_3$:MeOH:H_2O (60:45:5), the run time and the area of the peak is recorded. the average was calculated as shown in the

Table 3.13. Figure 3.15 is showing the analysis of hexane fraction on HPLC. Figure 3.16 is showing HPLC analysis of hexane fraction 0.1 ml (1 mg/ml) plus 0.1ml of **1**(1 mg/ml), the peak number 4 in this figure is for **1** because it be became bigger after addition 0.1 ml (1 mg/ml) of standard sample of **1** resulting from increase the concentration of **1**.

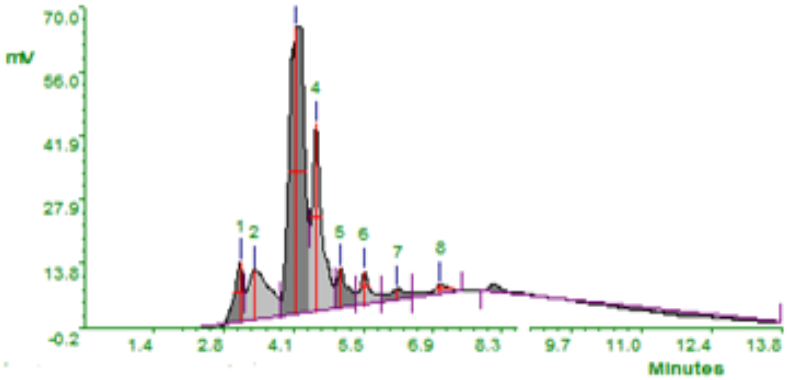


Figure 3.15 HPLC analysis of hexane fraction (1 mg/ml) of *S. europaea.*

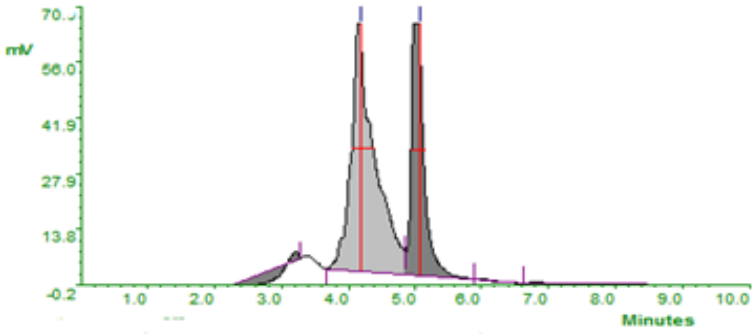


Figure 3.16 HPLC analysis of hexane fraction 0.1 ml (1 mg/ml) plus 0.1 ml of 1(1 mg/ml).

Table 3.13 Results of HPLC analysis of hexane fraction 0.1 ml (1 mg/ml).

Sample name	Area	Average
Hexane extract	49.23	36.74
Hexane extract	24.79	
Hexane extract	36.19	
Standard sample of **1**	32.52	34.05
Standard sample of **1**	38.13	
Standard sample of **1**	36.64	

Standard sample of **1**	32.98	

The average area of **1** standard sample with concentration (0.5 mg/ml) is 34.052, while the average area of **1** with concentration (x) in the hexane extract is 36.742 therefore X equal to 0.539 mg/ml. So each **1** mg of hexane extract contains 0.539 mg of **1,** therefore, 15 mg of hexane extract prepared from 100 g of *S. europaea* contains 8 mg of **1**. Furthermore, each (1g) of *S. europaea* contains (0.08 mg) of **1** (i.e. 0.0081 %).

3.4.4.3 Analysis of 1 in chloroform fraction of *S. europaea*

A total of 5 g of *S. europaea* was extracted at room temperature for two days with 80 % MeOH (3 × 50 ml) to yield (62.00 mg) of crude methanol extract which dissolved in (2 ml) of distilled water and partitioned three times with $CHCl_3$ (3 × 50 ml) to yield (5.3 mg) of $CHCl_3$ extract which dissolved in 5 ml of methanol for HPLC grad.

1 ml of chloroform extract (1mg/ml) was run on reverse phase HPLC column (10 × 150 mm, flow rate 2 ml/min., run time 30 min eluted with $CHCl_3$:MeOH:H_2O (60: 45:5), this run was repeated again with the same conditions except addition of 5 drops of **1**, therefore the tiny peaks for **1** (RT 4.88) in the first run become bigger after addition of drops from the standard compound in the second run as shown in the chromatogram below. Figure 3.17 chromatogram is showing HPLC analysis of *S. europaea* chloroform extract (0.5 mg/ml). Figure 3.15 HPLC analysis of *S. europaea* chloroform extract (0.5 mg/ml) plus 0.2 ml of 1(1 mg/ml).

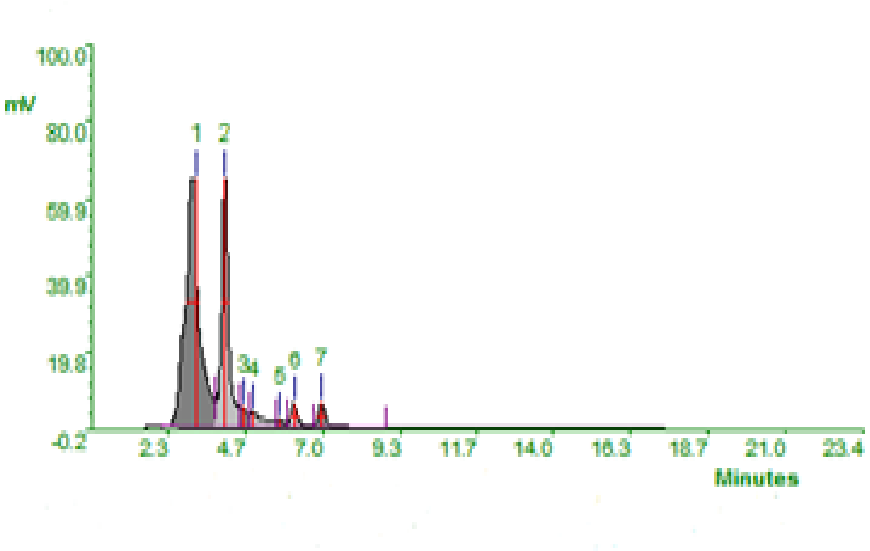


Figure 3.17 HPLC analysis of *S. europaea* chloroform extract (0.5 mg/ml).

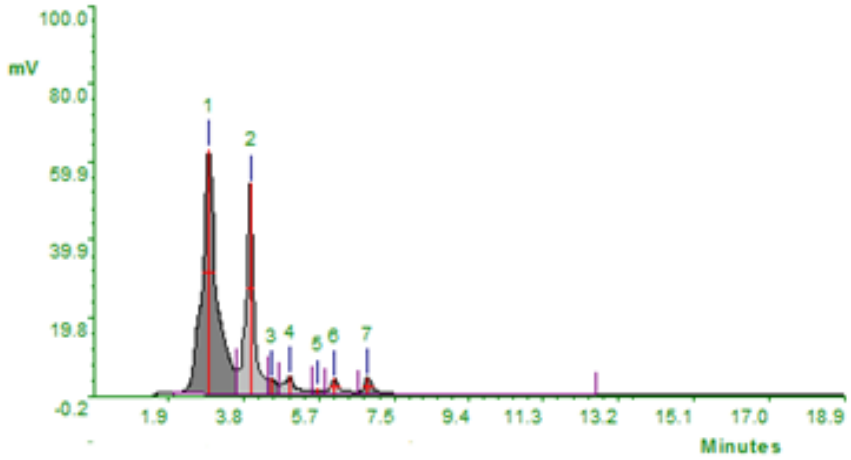


Figure 3.18 HPLC analysis of *S. europaea* chloroform extract (0.5 mg/ml) plus 0.2 ml of 1(1 mg/ml).

3.4.4.4 Analysis of 1 in crude glycosides of *S. europaea*

A samples of crude glycoside (B) was run on reverse phase silica gel column (10 × 225 mm, loop 100 µl) eluted with $CHCl_3$: MeOH:H_2O

(60:45:5). The concentration of **1** in the crude glycoside extract was found to be very small because only a tiny peak at the retention time of (5.1 min) was found. It was not possible to calculate the % **1** in the samples because the peaks were too small and also merged with glycoside peaks. Figure 3.19 chromatogram showing HPLC analysis of *S. europaea* crude glycosides sample B.

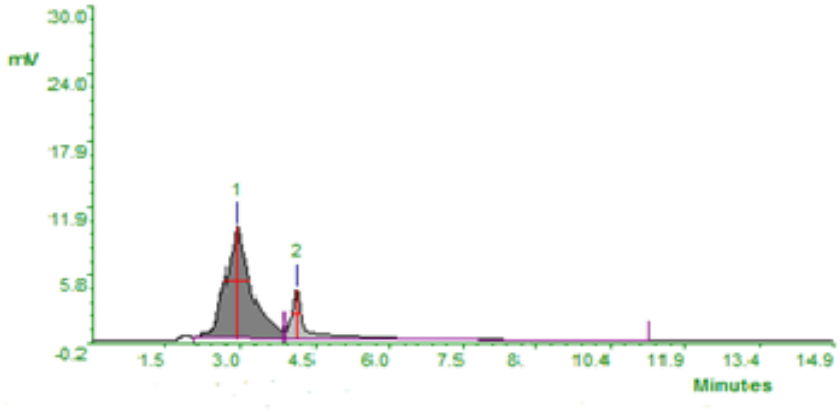


Figure 3.19 HPLC analysis of *S. europaea* crude glycosides sample B.

There are 4 possible sources in which **1** is present in the plant material as it can be synthesized by the plant itself, absorbed from the atmosphere or soil (plant habitat) or, it could be transferred to the plant during the extraction procedure (i.e. from the solvents or plastic equipment used to prepare the total plant extract) the plastic bags which were used to carry the plant to the research laboratory.

Isolation of **1** from the plant species was mentioned in several references such as, *Calotropis gigantean* (Habib and karim,, 2009), *Alchornea cordifolia* (Mavar et al., 2008), *Aloe vera* (Lee K. F. et al., 2000), *Euphorbia cyparissias* and *Euphorbia seguieriana* (Tothsoma et al., 1993). *Ricinus communis* (*Euphorbiaceae*) (Pateh, 2009), Nuclea officinalis leaves (Kui, 2009), culture broth of a fungus strain No. 36 *Pencillium olsonii* (Amade et al., 1994) and *Phyllanthus niruri*. Marine *Sadiment* (at percentage 14.13%) (SudhaSrikesavan and Selvam, 2012),

Nocardia levis MK-VL_113 & Streptomyces tendae TK-VL_333 (Alapati and Muvva, 2013).

In the following species the quantity or percentage of **1** in the plant extract was high, Marine macro alga, *Ulva fasciata* with percentage of **1** (88.42%), *Calotropis gigantean* (1.0 kg of plant material yield 38 g crude ethyl acetate extract of which 10 g was fractionated on normal phase silica gel for column chromatography and preparative TLC to yield 98 mg of **1**). Compound **1** can be produced naturally by many types of algae and fungus. Chen (2004) reported the biosynthesis of 1 by red algae (*Bangia atropurpurea*) grown in a culture medium containing 14C-labeled sodium bicarbonate (Chen, 2004).

Namikoshi *et al.,* (2006) calculated the modern carbon content of **1** isolated from three different algae (2 of which are edible) to range from 50% to 87% (Namikoshi et al., 2006). Amade *et al* (2009), isolated two metabolites from culture broth of a fungus strain No. 36 *Pencillium olsonii* (30 liters of *Pencillium olsonii* was used, first fraction 1.8 g was loaded on a silica gel column, fraction eluted with hexane – EtOAc (900 mg) was further purified to yield 725 mg of **1** (Amade *et al*., 1994).

There is no clear mechanism by which **1** is absorbed from the atmosphere or soil (i.e. how much of **1** can be transfer to the plant) therefore it is difficult to make the decision about the sources of **1**.

In the plant under investigation (S*. europaea*) it can be eliminate the possibility of transfer **1** to the plant during the extraction process is unlikely because plastic equipment was not used during the extraction process. Also 2.5 L of hexane (analytical grade reagent) was dried using rotary evaporator then TLC analysis of the residue and a sample of standard **1** was carried out. The results revealed that the residue did not contain **1**, therefore this eliminates the possibility of source of **1** as a solvent contamination. Further work needs to be carried out with respect to

extraction of a sample of the soil in which the *S. europaea* is growing (a sample of soil in Dr Wright's Garden). Moreover, growing of *S. europaea* in an a special atmosphere which supplied it only with oxygen then carried out the extraction of a sample from this source and producing TLC analysis of the total extract and a sample of **1** is required to make a decision about the sources of **1**.

3.4.5 Isolation of palmitic acid 2.

A total of 74 mg hexane extract was separated on a normal silica gel column (G 60 F_{254} nm, mesh 200-400 µm, 2.5 × 40 cm) eluted with n-hexane:dichloromethane gradient, followed by increasing the polarity using dichloromethane:ethyl acetate gradient as illustrated in Table 3.14.

Table 3.14 Solvent conditions of silica gel column for isolation of 2.

Mobile phase	% of compositions	Volume (ml)	Fraction Number(weights not recorded)
Hexane	100	100	1-6
Hexane: Dichloromethane	95:5	100	7-10
Hexane: Dichloromethane	92:7	100	11-14
Hexane: Dichloromethane	90:10	100	15-17
Hexane: Dichloromethane	85:15	100	18-20
Hexane: Dichloromethane	80:20	100	21-24
Hexane: Dichloromethane	70:30	100	25-27
Hexane: Dichloromethane	60:40	100	28-31
Hexane: Dichloromethane	50:50	100	32-35
Hexane: Dichloromethane	40:60	100	36-39
Hexane: Dichloromethane	30:70	100	40-45
Hexane: Dichloromethane	20:80	100	46-49
Hexane: Dichloromethane	10:90	100	50-53
Hexane: Dichloromethane	100	100	54-57
Dichloromethane:ethyl acetate	90:10	100	58-61
Dichloromethane:ethyl acetate	80:20	100	62-65
Dichloromethane:ethyl acetate	70:30	100	66-69
Dichloromethane:ethyl acetate	60:40	100	70-73
Dichloromethane:ethyl acetate	50:50	100	74-77

3.4.5.1 Further purification of 2

The TLC results (silica gel 60 F_{254} aluminium sheets 20× 20 cm plates, chloroform: methanol: water (60:40:10) as a developing solvent and vallinin sulphuric acid as a spray reagent) of fraction (62-73) table (3.14) revealed that it has one component plus a small quantity of impurity. Therefore it was further purified on normal phase silica gel column. The column conditions are summarized in Table 3.15. The TLC analysis of collected fractions showed that fractions (34-38) 2 mg have a single spot of **2**.

Table 3.15 Conditions of silica gel column chromatography of further purification of 2.

Mobile phase	% of solvents	Volume (ml)	Fractions number
Hexane	100	100	1-5
Hexane: Dichloromethane	98:2	100	6-8
Hexane: Dichloromethane	94:6	100	9-11
Hexane: Dichloromethane	92:8	100	12-14
Hexane: Dichloromethane	90:10	100	15-17
Hexane: Dichloromethane	80:20	100	18-20
Hexane: Dichloromethane	70:30	400	21-30.
Hexane: Dichloromethane	60:40	300	31-40 (Compound **2**)
Hexane: Dichloromethane	50:50	100	41—44
Hexane: Dichloromethane	40:60	100	45-47
Hexane: Dichloromethane	30:70	100	48-50
Hexane: Dichloromethane	20:80	100	51-53
Hexane: Dichloromethane	10:90	100	54-56
Dichloromethane:ethyl acetate	90:10	400	57-63.

TLC analysis (section 2.5.5.) indicated that fractions (34-38, 2mg) have a single spot of **2**.

3.4.5.2 Structure elucidation of 2

Compound **2** was isolated as oily substance from the hexane extract of *S. europaea.* TLC analysis (section 2.5.5.) of **2** showed no UV absorbtion at

254 or 357 nm and a dark blue colour after spraying with vanillin sulphuric acid and heating the plate at 200 C^{O}.

The ES- mass spectrum of **2** Figure 3.20 showed a molecular ion peak [M-1] at m/z = 255.4, other peaks were observed at 256.4 [M^{+}], 512.6 [$2M^{+}$], 537.6 [2M+Na+2H]. Accurate mass spectrum [EPSRC National Facility Swansea, LTQ Orbitrap XL] Figure 3.21 showed a significant peak at 257.2 [M+1] suggesting the molecular formula $C_{16}H_{32}O_2$. Figure 3.22 an expansion of accurate mass spectrum of **2**.

The ^{1}H NMR and ^{13}C NMR data of **2** are shown in
Table 3.16. The ^{13}C NMR (400 MHz, $CDCl_3$) spectrum of **2** Figure 3.23 displayed signals at 179.7 (C-1), 34 (C-2), δ between 28 to 30 are CH_2 type for (C-3 to C-14), 22 (C-15, CH_2 α to CH_3), 24.5 (C-15 β to CH_3), 14.13 (C-16, CH_3). Figure 3.24 an expansion of ^{13}CNMR (400 MHz, $CDCl_3$) spectrum of **2**.

The DEPT (400 MHz, $CDCl_3$) spectrum of **2** Figure 3.25 shows peak at δ 14.13 C-16, CH_3), other peaks δ between 28 to 30 are CH_2 type for (C-3 to C-14), these data are in accordance with reported data (Joshi et al., 2009). Figure 3.26 expansion of DEPT (400 MHz, $CDCl_3$) spectrum of **2**.

The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **2** Figure 3.27 showed signals at 2.60 (2H, t, H-2), 1.25-1.30 (24H, m, H-3 to H-14), 1.57 (1H, m, α H-15), 2.17 (1H,m, β H-15) and 0.84 (3H, t, H-16). Figure 3.28 expansion of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 2.

Table 3.16 ^{1}H and ^{13}C NMR and DEPT data (400 MHz, $CDCl_3$) of 2.

Carbon number	^{1}H NMR data (400 MHz, $CDCl_3$)	DEPT	^{13}C NMR data (400 MHz, $CDCl_3$)
1	-	O-C=O	179.7
2	2.60 (2H, t, H-2)	CH_2	34
3-14	1.25-1.30 (24H, m, H-3 to H-14).	12 × CH_2	28 to 30
15	1.57 (1H,m, α H-15) 2.17 (1H,m, β H-15)	CH_2	22.71 24.5
16	0.84 (3H, t, H-16)	CH_3	14.13

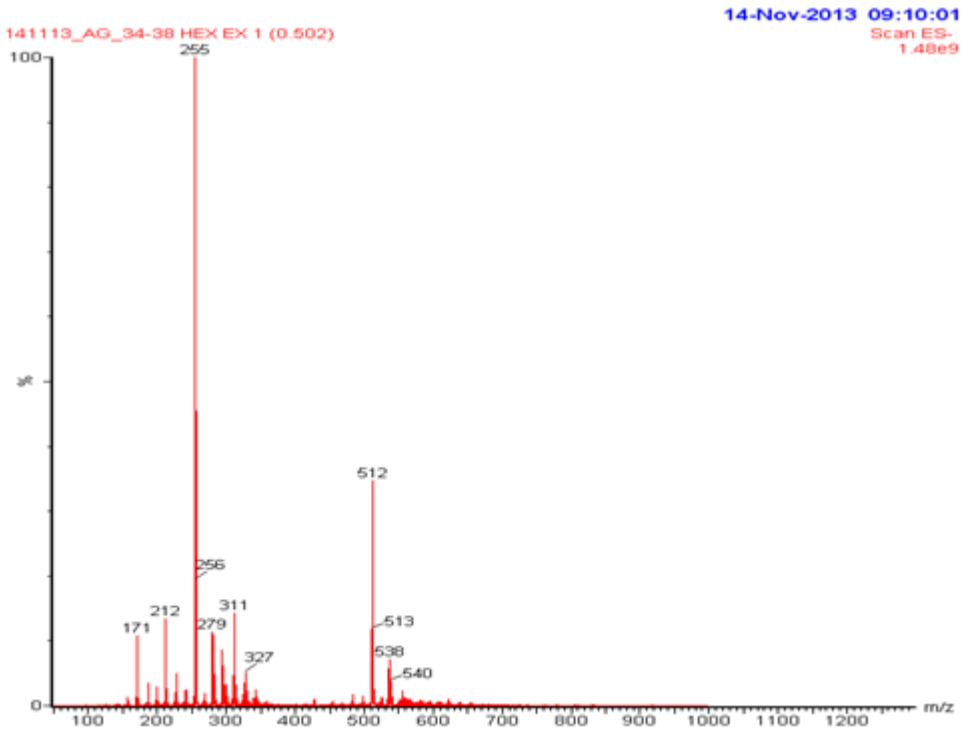


Figure 3.20 ES- mass spectrum of 2.

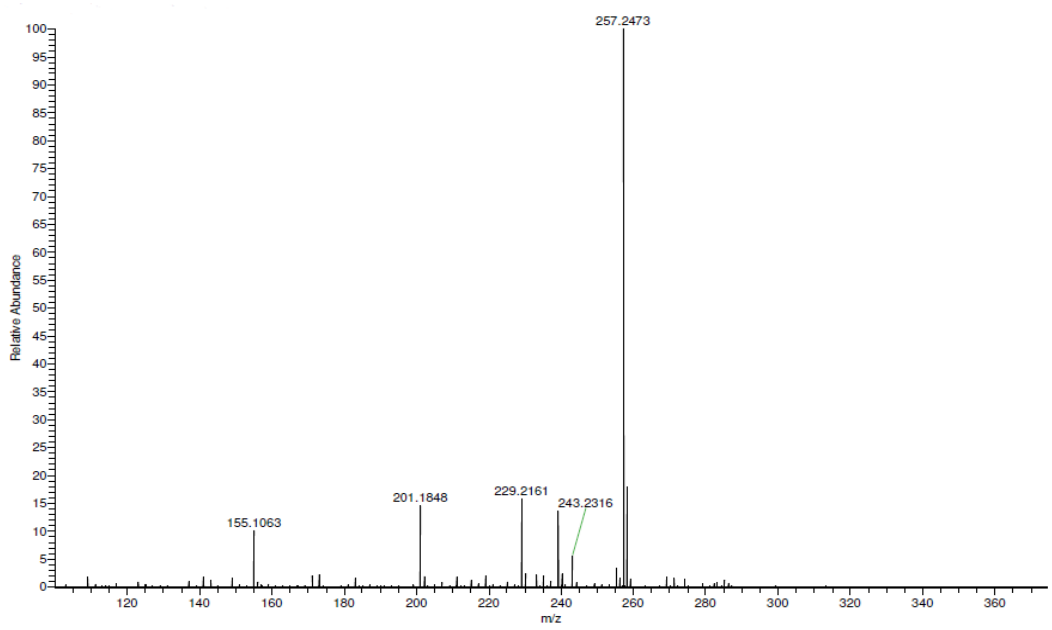


Figure 3.21 Accurate mass spectrum of 2.

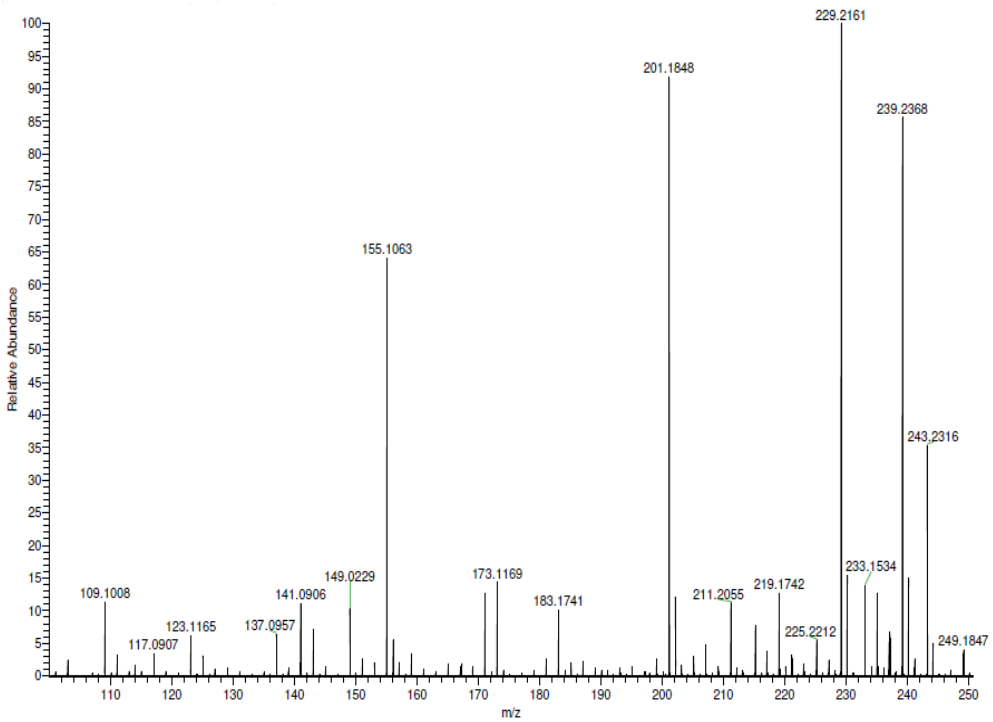


Figure 3.22 Expansion of accurate mass spectrum of 2.

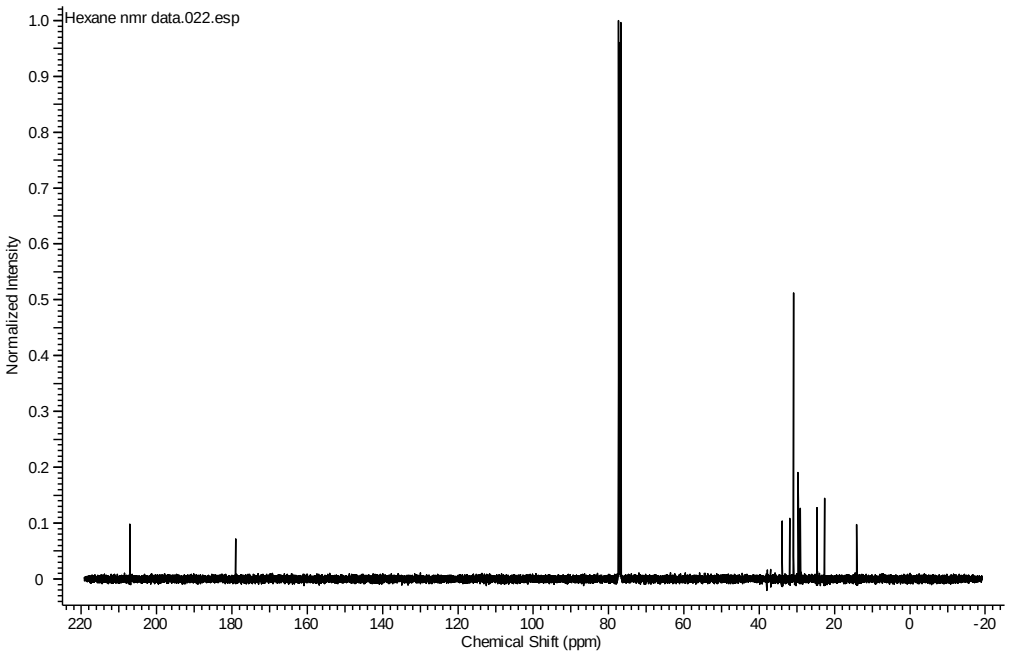


Figure 3.23 ^{13}CNMR spectrum (400 MHz, $CDCl_3$) of 2.

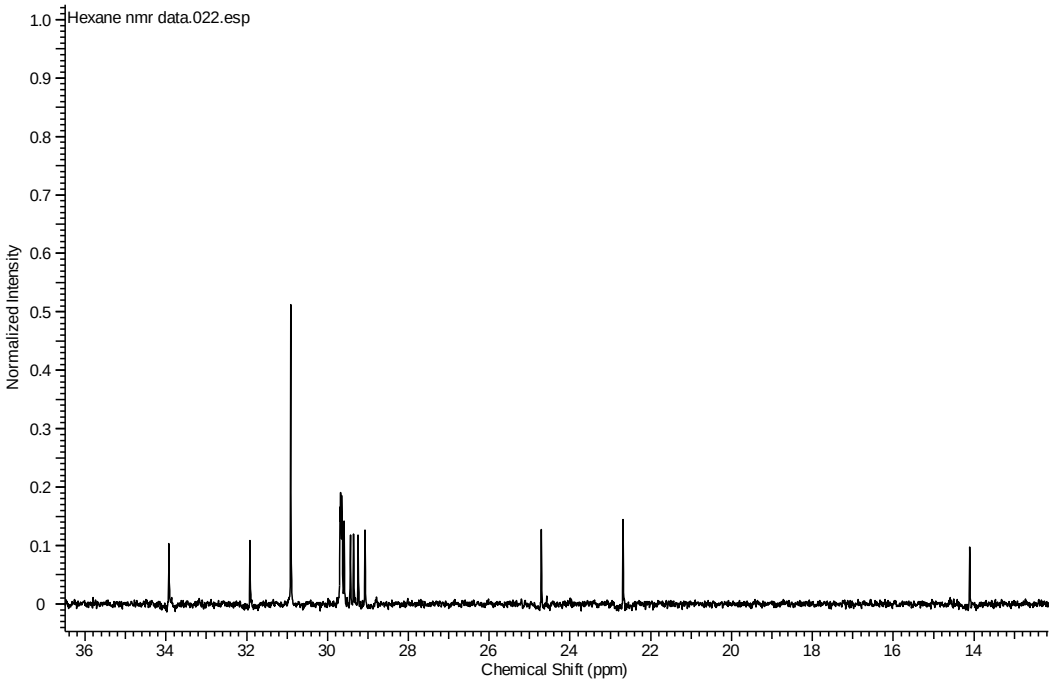


Figure 3.24 Expansion of ^{13}CNMR spectrum (400 MHz, $CDCl_3$) of 2.

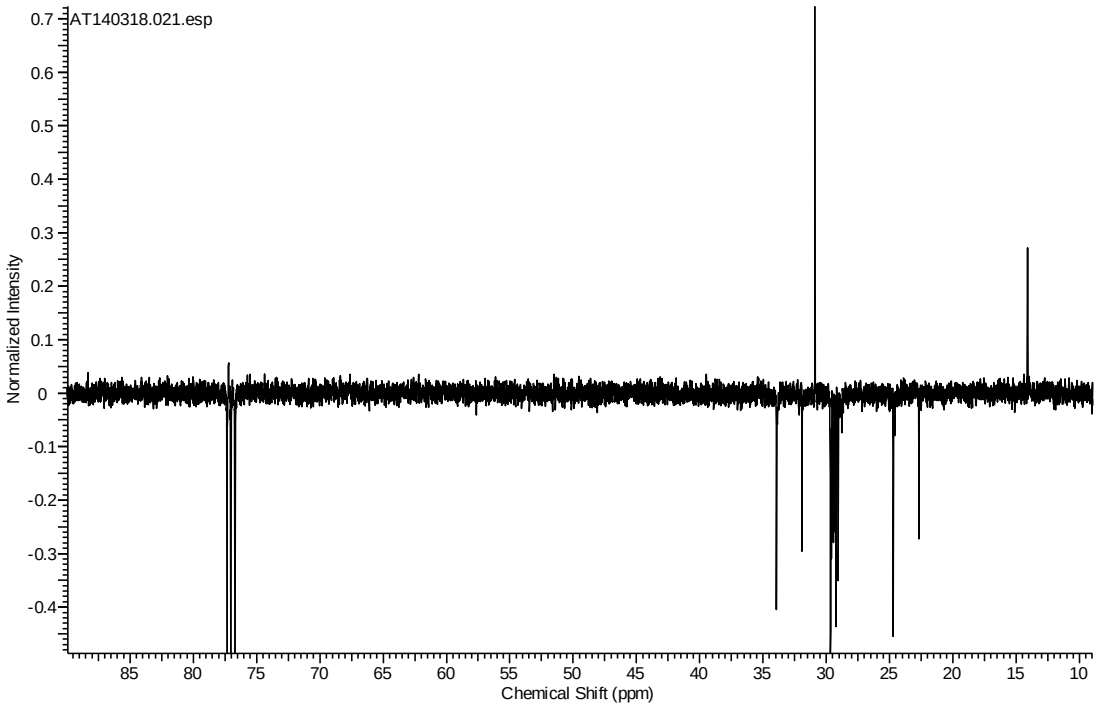


Figure 3.25 DEPT spectrum (400 MHz, $CDCl_3$) of 2.

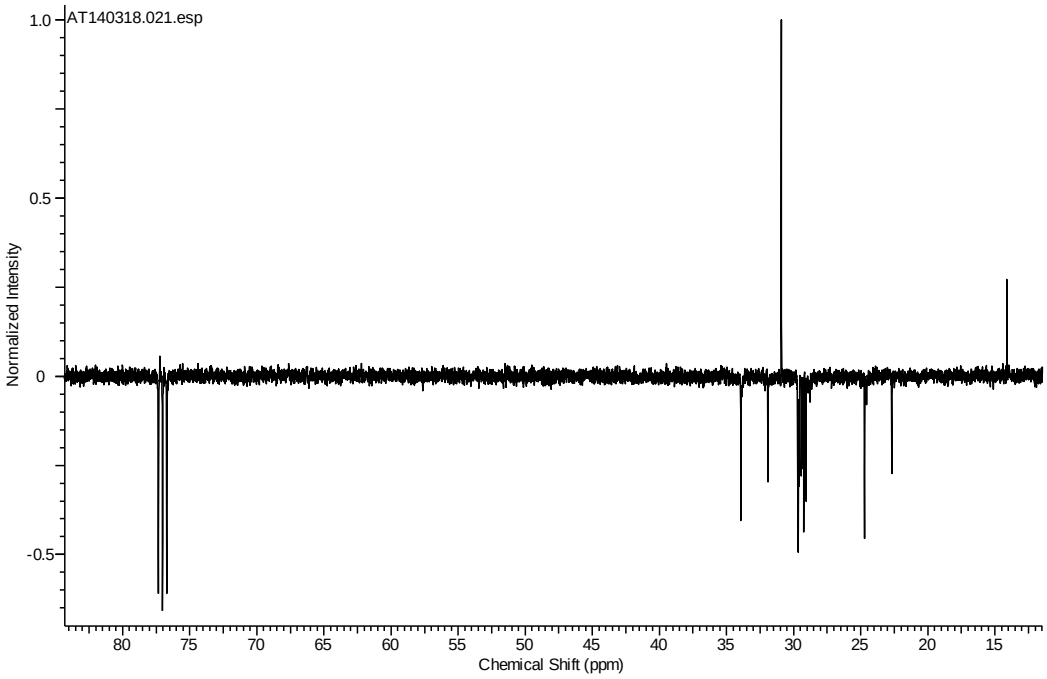


Figure 3.26 Expansion of DEPT spectrum (400 MHz, $CDCl_3$) of 2.

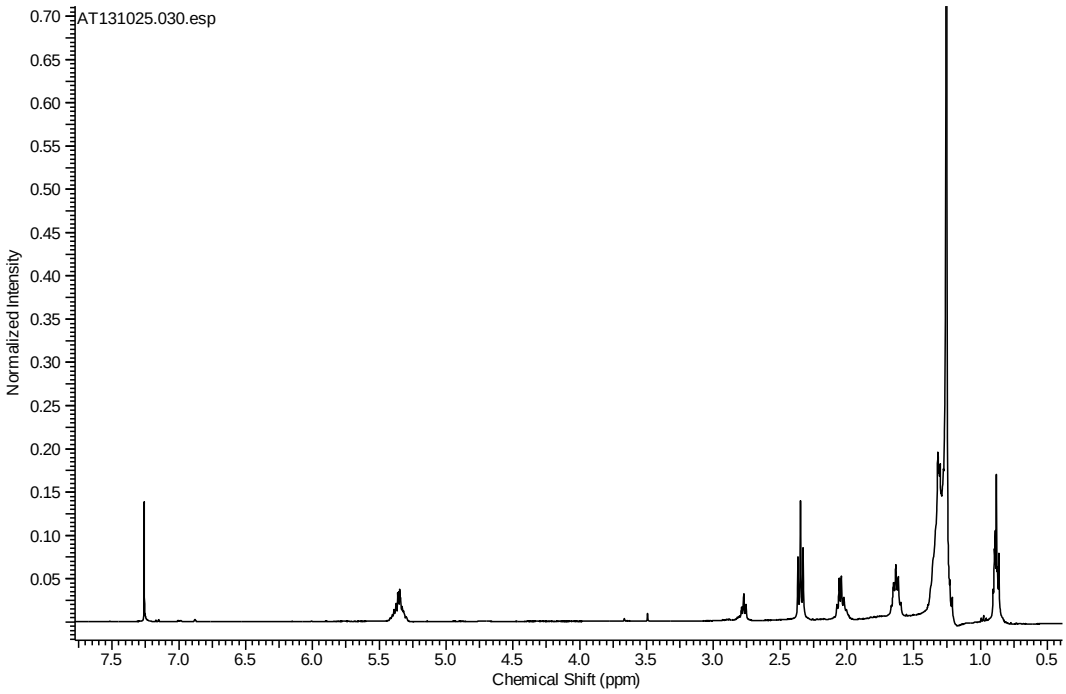


Figure 3.27 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 2.

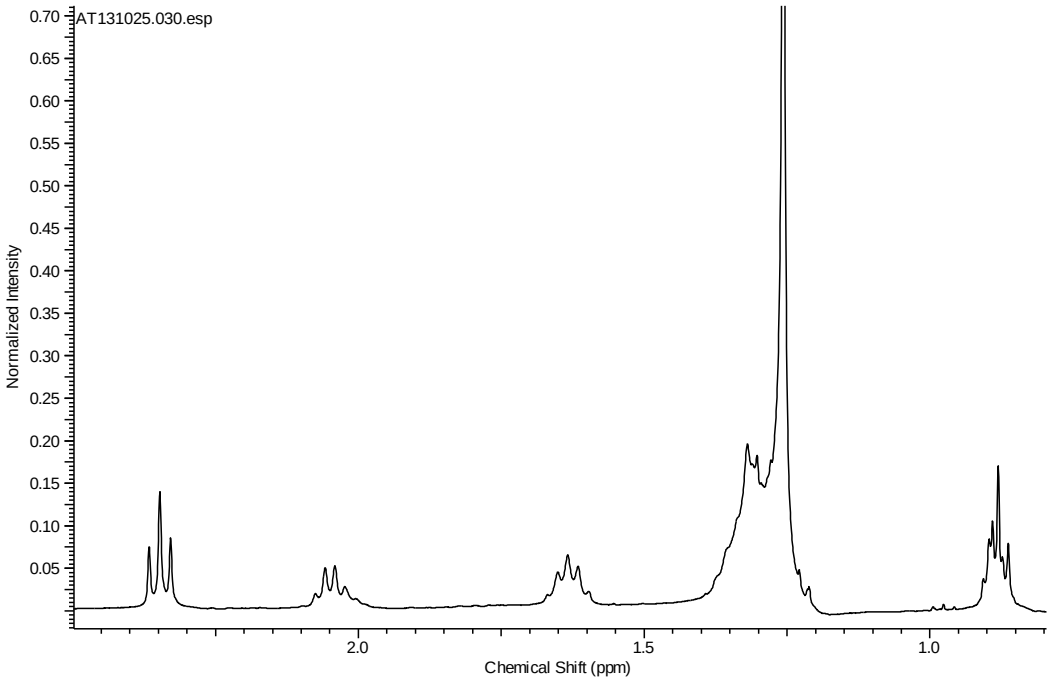


Figure 3.28 Expansion of ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 2.

Palmitic acid **2** was first isolated from palm oil in 1800 S, it also reported in other foods such as cheese, meat, butter, milk and olive oil. **2** is present in chocolate beside oleic and stearic acids.

Harada *et al* (2002) studied the cytotoxic activity of **2** isolated from marine red alga. The results revealed that **2** has selective antitumor activity to human leukemic cells, but no cytotoxicity to normal HDF cell's at concentrations of 12.5 to 50 µg/ml (Harada *et al*., 2001).

It has been reported that **2** stimulates glucose incorporation in the adipocyte by mechanism likely involving intracellular calcium (Thode *et al*., 1989).

Joshi-Barve *et al* (2007) reported that patients with non-alcoholic steatohepatitis (NASH) have elevated serum levels of proinflammatory cytokines, such as interleukin-8 (IL-8), which are likely to contribute to hepatic injury. Therefore they examined the effect of hepatic steatosis on IL-8 PRODUCTION. They induced lipid accumulation in hepatocytes (Hep G_2, rate primary hepatocytes and human primary hepatocytes) by exposing them to pathophysiologically relevant concentrations of palmitic acid to simulate the excessive influx of fatty acids into hepatocytes.

Significant fat accumulation was documentd morphologically by Oil Red O staining in cells exposed to palmitic acid and it was accompanied by an increase in intracellular triglyceride levels. Palmitic acid was found to induce significantly elevated levels of biologically active neutrophile chemoattractant, IL-8, from steatotic hepatocytes (Joshi‐Barve *et al*., 2007).

3.4.6 Isolation of Rosmarinic acid 3.

A total of 26 mg of ethyl acetate fraction of the methanolic extract was fractionated over Sephadex LH20 (2.5 x 40 cm) (section 2.5.4), eluted with (300 ml, MeOH (100%). A total of 22 fractions of 21 ml each were collected. TLC analysis of the fractions (section 2.5.5.) revealed that fractions (20-21) 10 mg composed from one compound with some impurities, Sephadex chromatography was repeated for this fraction, eluting with (300ml, MeOH (100%) and a total of 19 fractions (21) ml each were collected. TLC analysis revealed that sub fractions 12-14 (20-21) 3 mg and 15-17 (20-21) 1 mg appeared to contain individual compounds. Fraction (18-19) 7 mg has a single compound with small impurity; therefore it was again subjected to Sephadex chromatography with the same conditions. A total of 23 fractions (7 ml each) were collected, sub fractions (22-23, 1 mg) and (12-21, 2 mg) have a single spot. TLC analysis (section 2.5.5.) were carried out for all the single compounds, they are found the same, and therefore they are collected to yield 7 mg of 3.

3.4.6.1 Structure elucidation of 3

Compound **3** (Rosmarinic acid) was separated as a sticky compound (7 mg) from the ethyl acetate fraction of the methanolic extract of *S. europaea* TLC analysis (section 2.5.5.) of **3** was visible at 254 nm with a dark blue spot before spraying, while a pink spot after spraying. **3** showed a dark blue spot after spraying with ferric chloride, which indicates that it is a phenolic compound. **3** did not show any colour with Dragendorff's reagent; suggests that it is not an alkaloid.

The ES+ mass spectrum of **3** Figure 3.31 showed a molecular ion peak $[M^+]$ at *m/z* = 360.3, another peak was observed at m/z = 383.1 $(M + Na)^+$. Figure 3.32 ES- mass spectrum of **3**.

The ^{1}H NMR and ^{13}C NMR data of **3** are shown in Table 3.17. The ^{13}C NMR spectrum (600 MHz, CD_3OD) of **3**

Figure 3.33 shows 18 signals, therefore it has 18 carbon atoms, 2 of them are carbonyl carbons because they appear at δ ≈ 170 ppm. The most important peaks characteristic to C-9a, δ = 166, C-9b, δ = 170.1, other peaks were at 125.4 (C-1a), 113.9 (C-2a), 145.6 (C-3a), 146.6 (C-4a), 115.2 (C-5a), 121.9 (C-6a), 144.8 (C-7a), 113.1 (C-8a), 127 (C-1b), 116.3 (C-2b), 145.0 (C-3b), 144.0 (C-4b), 115.4 (C-5b), 120.2 (C-6b), 36.6 (C-7b), 73.4 (C-8b). Figure 3.34 expansion of ^{13}CNMR spectrum of **3** (600 MHz, CD_3OD).

The DEPT spectrum Figure 3.35 shows one ($-CH_2-$) group, nine (-CH-) groups ($9 \times CH = C_9H_9$) and eight carbon atoms ($8 \times C = C_8$) two of them are carbonyl carbons, therefore this accounts for $C_{18}H_{11}$, and suggest 5-OH groups are present.

The ^{1}H NMR (400 MHz, CD_3OD) spectrum of **3** Figure 3.36 showed the existence of two groups of H-atoms in ABX structure on 1,3,4-trisubstituted benzene rings [δ 7.03 (1H, *d, J* = 2.0 Hz, H-2a) / 6.76 (1H, *d, J* = 8.2 Hz, H-5a) / 6.93 (1H, *dd, J* = 2.0 Hz, H-6a); δ 6.73 (1H, *d, J* = 2.0 Hz, H-2b) / 6.65 (1H, *d, J* = 8 Hz, H-5b) / 6.64 (1H, *d, J* = 8 Hz, H-6b)]. Two benzoic methane signals [δ 7.54 (1H, *d, J* = 16 Hz, H-7a) / 6.25 (1H, *d, J* = 16 Hz, H-8a)]; two signals for aliphatic protons [δ 3.0 (2H, m, H-7b) / 5.00 (1H, m, H-8b)] were also observed in the spectrum. Figure 3.36 expansion of ^{1}HNMR spectrum of **3** (600 MHz, CD_3OD). Figure 3.29 part of structures of **3** assigned from ^{1}H NMR spectrum.

The correlations between H-2a / H-7a, H-2b / H-7b in the long range ^{1}H-^{1}H COSY spectrum Figure 3.38 indicated the linkages of C-1a / C-7a, C-1b / C-7b respectively. Figure 3.30 part of structures of **3** assigned from H-H Cosy and HMBC spectra. Figure 3.39 shows HMQC NMR spectrum of **3** (600 MHz, CD_3OD).

In the HMBC spectrum of **3** Figure 3.40 there is a long rang correlation between H-7a and H-8a. Also H-8a is correlated with C-1a which already correlated with H-7a indicating that C-7a and C-8a are connected with each other.

In the HMBC H-7a is correlated with C-9a (quaternary carbon δ 166 also H-8a is correlated with the same carbon C-9a his allows the placement of the carbonyl group. from the ^{1}H-^{1}H COSY it is indicated that C-1b at δ = 127.3 is connected to C-7b which has correlation with C-6b (δ = 120.2) / C-2b δ = 116.7 and another carbon at δ = 170.1 which theoretically could be a carbonyl group, from HMBC spectrum there is a single at (δ = 5.0 m) correlated to C-1b and to single at (δ = 170.1 which could be a carbonyl group) as long as C-7b is connected to C-1b this confirm that C-7b is attached to a newly assigned carbon C-8b and the latter is attached to the carbonyl group at δ = 170.1.

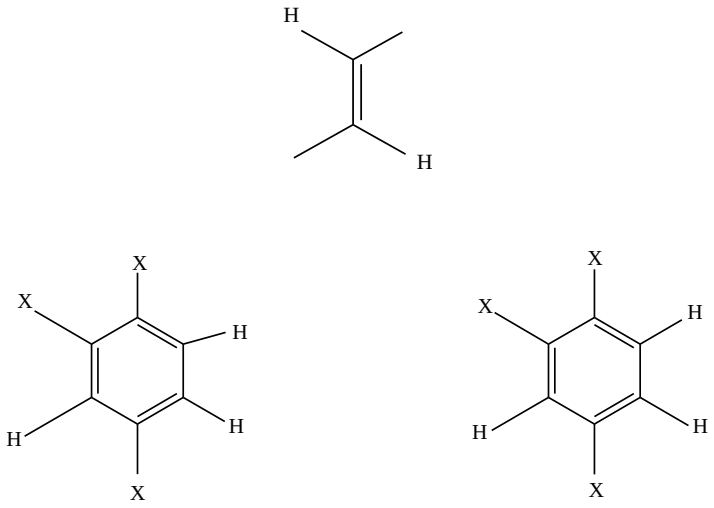


Figure 3.29 Partial structures of 3 assigned from ^{1}H NMR spectrum.

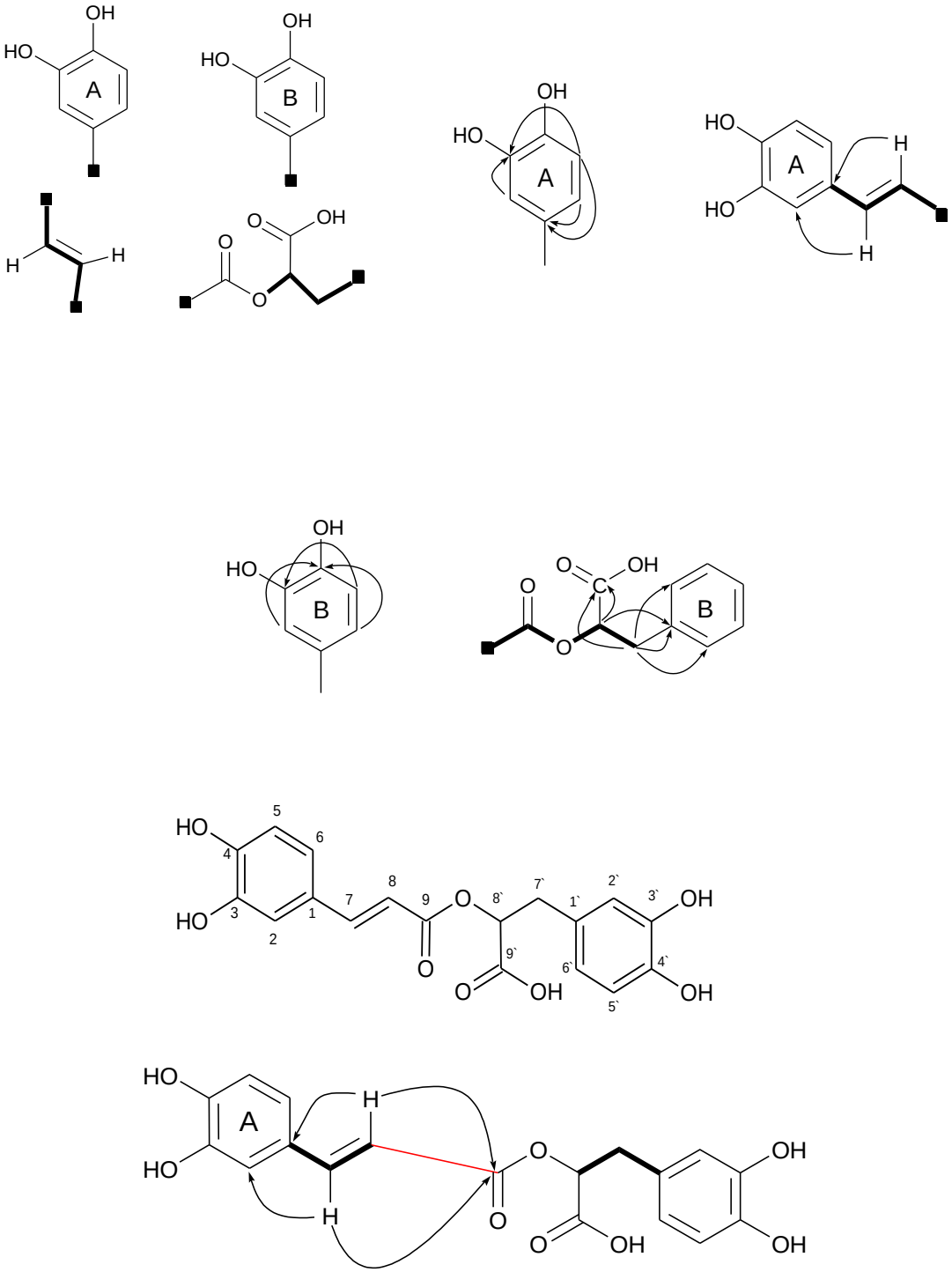


Figure 3.30 Partial structures of 3 assigned from H-H COSY (bold bond) and HMBC (arrows) spectra.

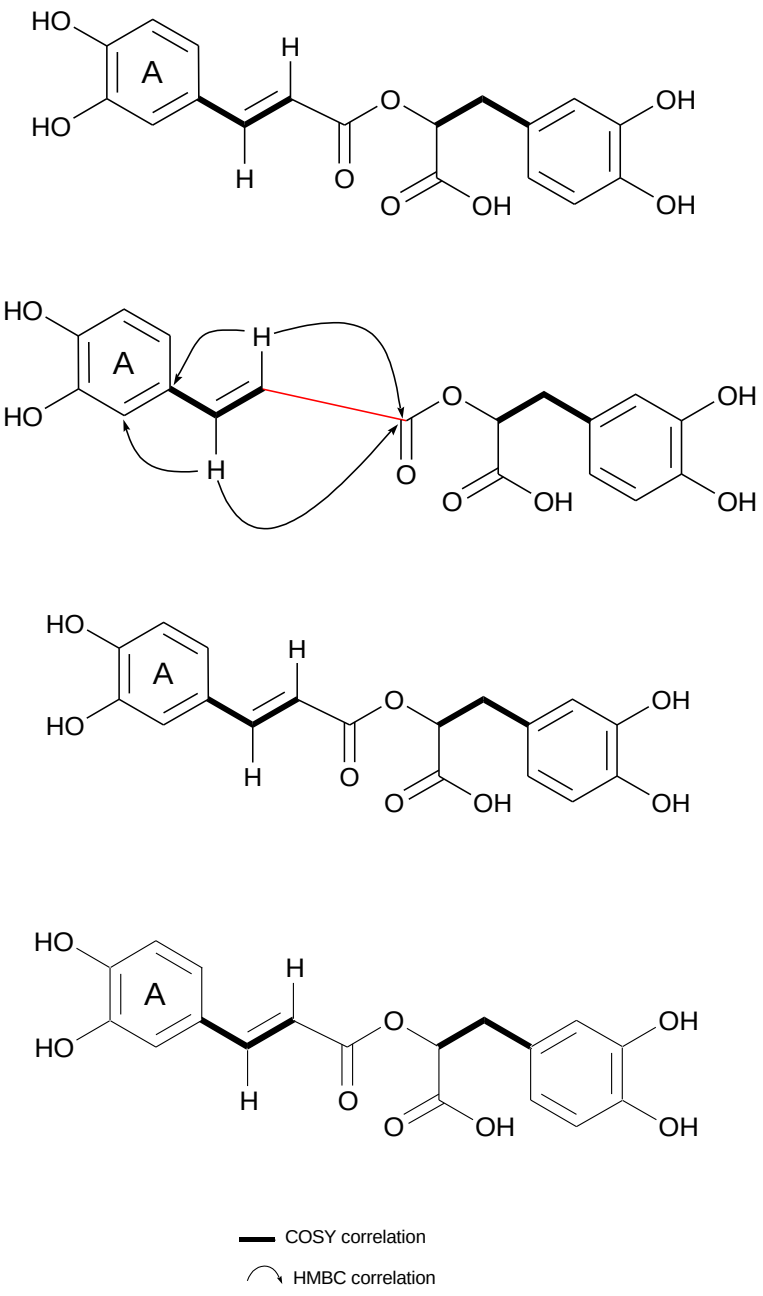


Figure 3.30 Continued.

Table 3.17 1D and 2D NMR date (600MHz, CD_3OD) of 3.

Carbon No.	δ ^{1}H NMR data (600 MHz, CD_3OD)	δ ^{13}C NMR data (600 MHz, CD_3OD)	HMBC
1	-	125.4	-
2	7.03 (1H, d, *J* = 2.0, H-2)	113.9	125.4, 148.6
3	-	145.6	-
4	-	146.6	-
5	6.76 (1H, d, *J* = 8.2, H-5)	115.8	125.4, 145.6
6	6.93 (1H, dd, *J* = 2.0, 8.2, H-6)	121.9	148.6
7	7.54 (1H, d, *J* = 16, H-7)	144.8	125.4, 113.9, 166
8	6.25(1H, d, *J* = 16, H-8)	113.1	125.4, 166
9	-	166	-
1`	-	127	-
2`	6.73 (1H, dd, *J* = 2.0, H-2`)	116.3	120.2, 144.0
3`	-	145.0	-
4`	-	144	-
5`	6.65 (1H, d, *J* = 8, H-5`)	115.4	127.3, 145.0
6`	6.54 (1H, d, *J* = 8, H-6`)	120.2	116.7, 144.0
7`	3.0 (1H, m, H-7`)	36.6	116.7, 120.1, 127.3, 170.1
8`	5.00 (1H, m, H-8`)	73.4	127.3, 170.1
9`	-	170.1	-

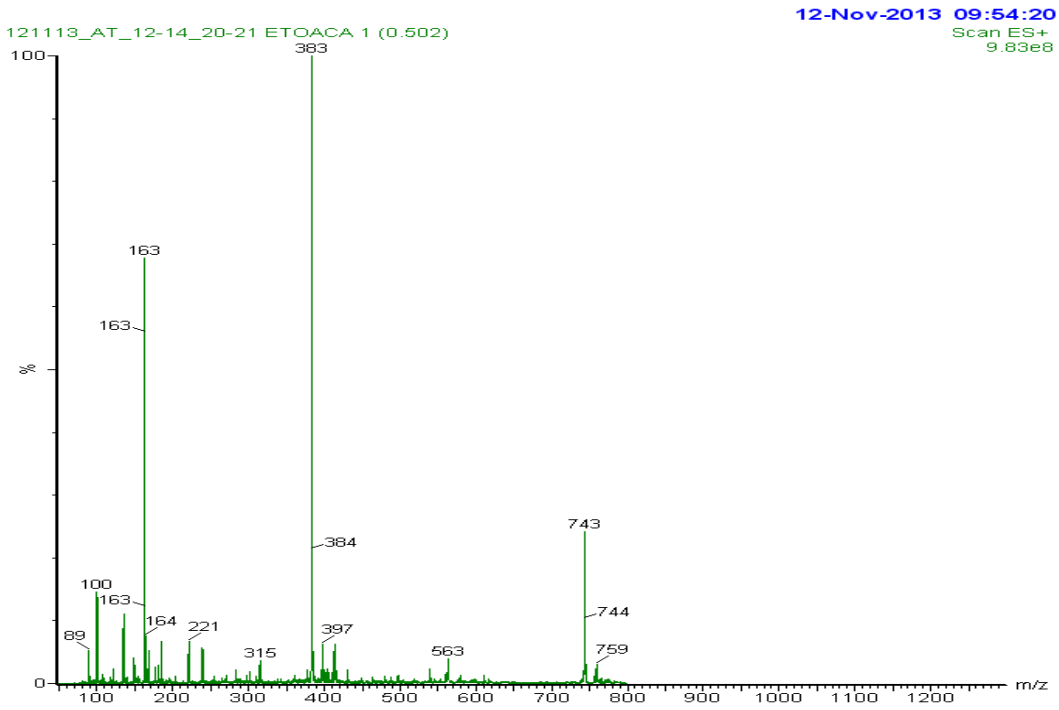


Figure 3.31 ES+ mass spectrum of 3.

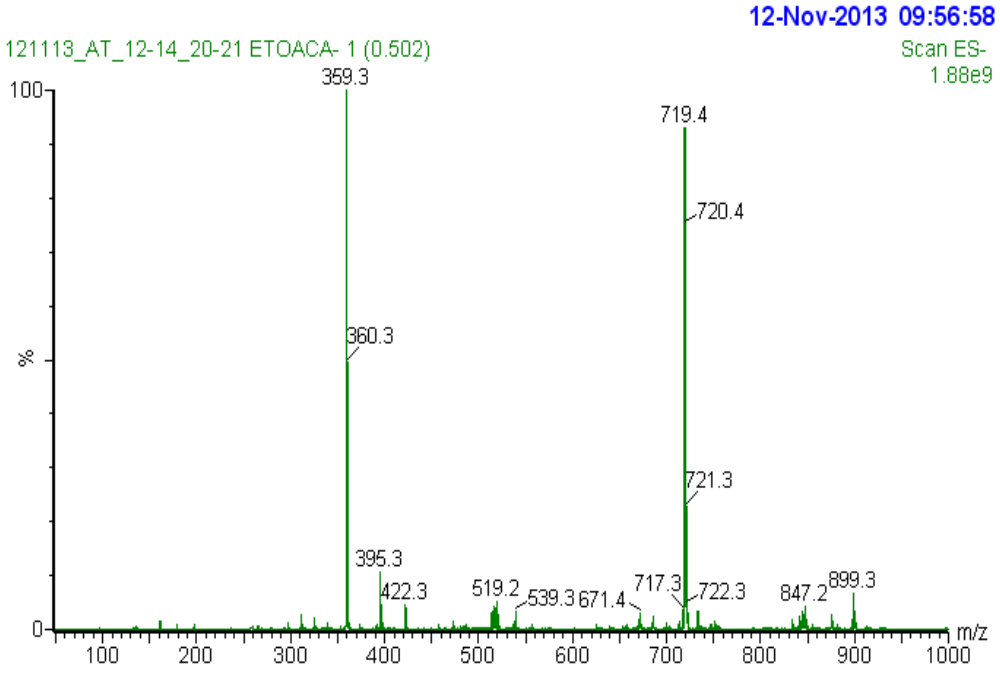


Figure 3.32 ES- mass spectrum of 3.

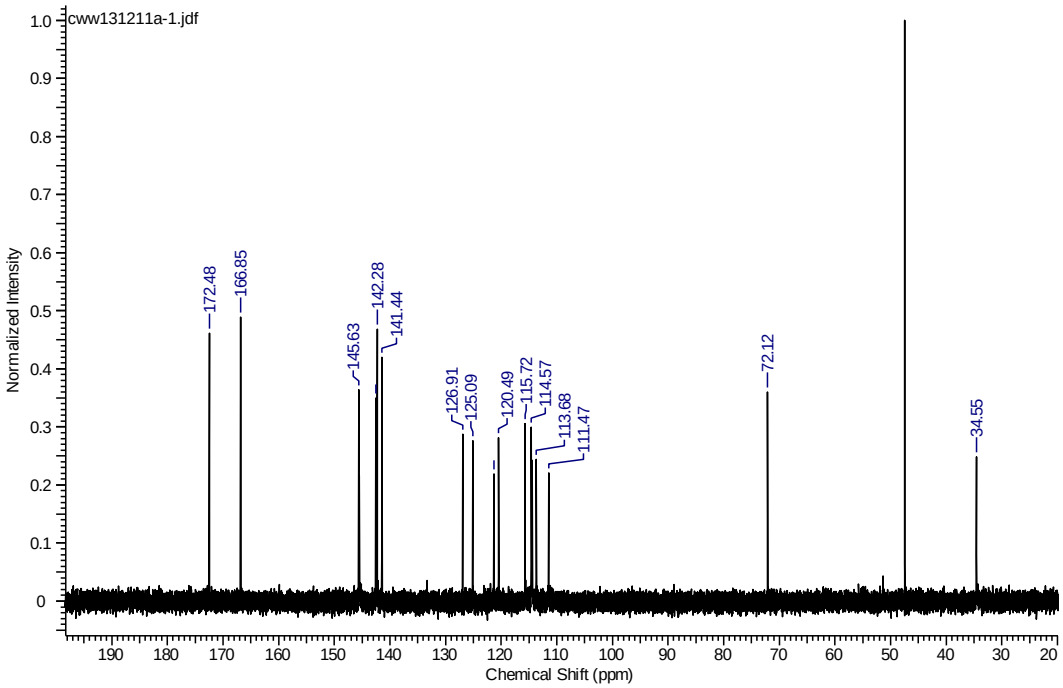


Figure 3.33 ^{13}CNMR spectrum (600 MHz, CD_3OD) of 3.

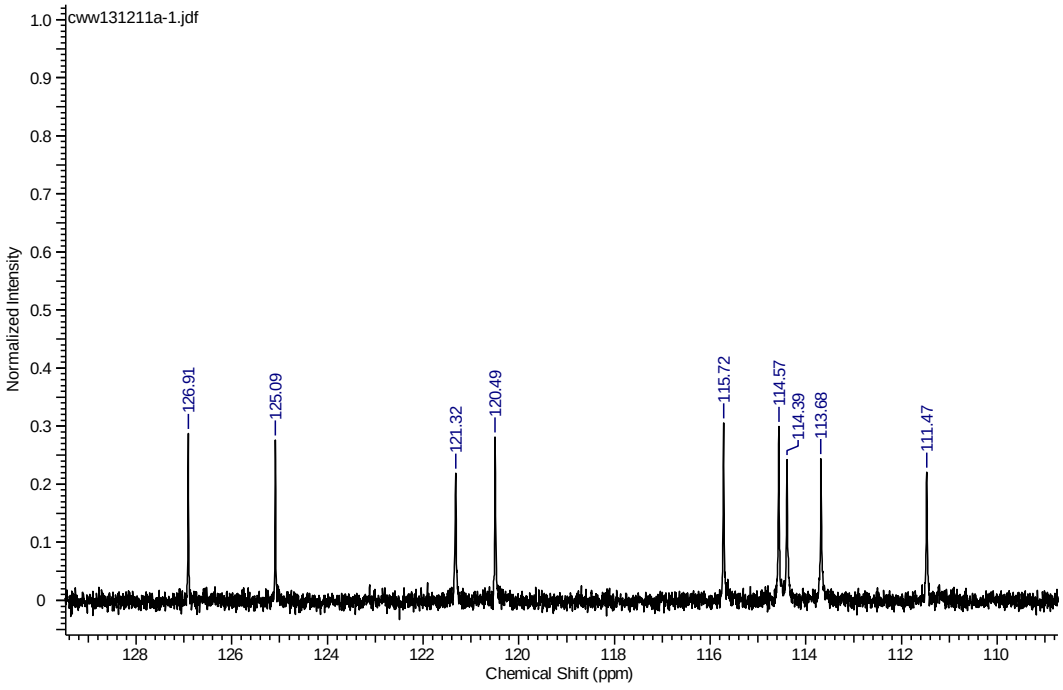


Figure 3.34 Expansion of ^{13}CNMR spectrum (600 MHz, CD_3OD) of 3.

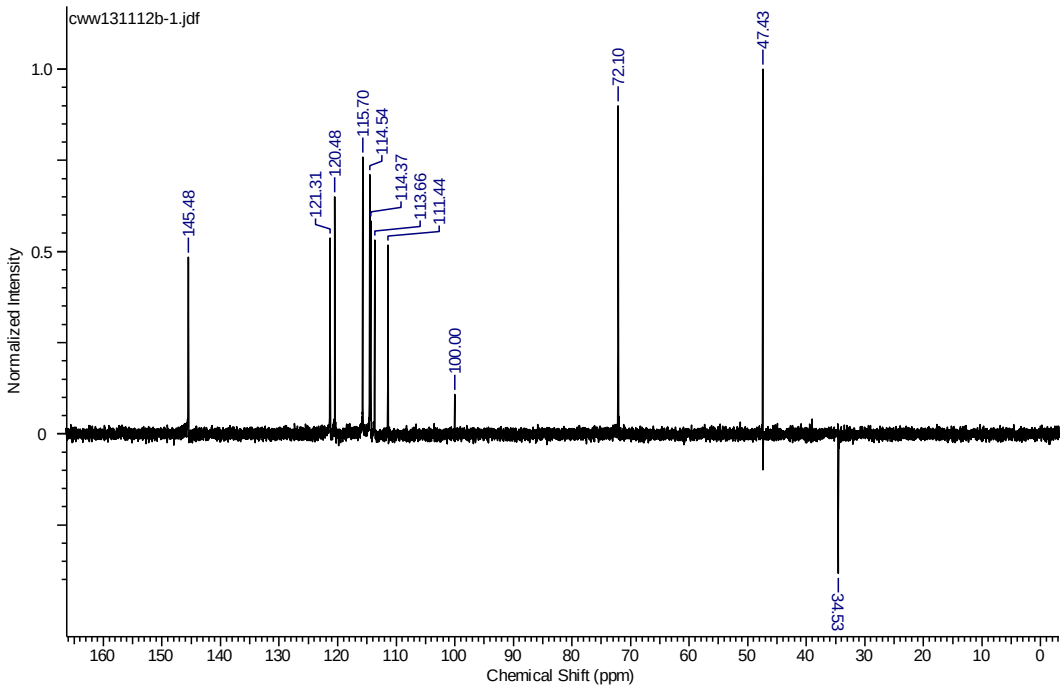


Figure 3.35 DEPT spectrum (600 MHz, CD_3OD) of 3.

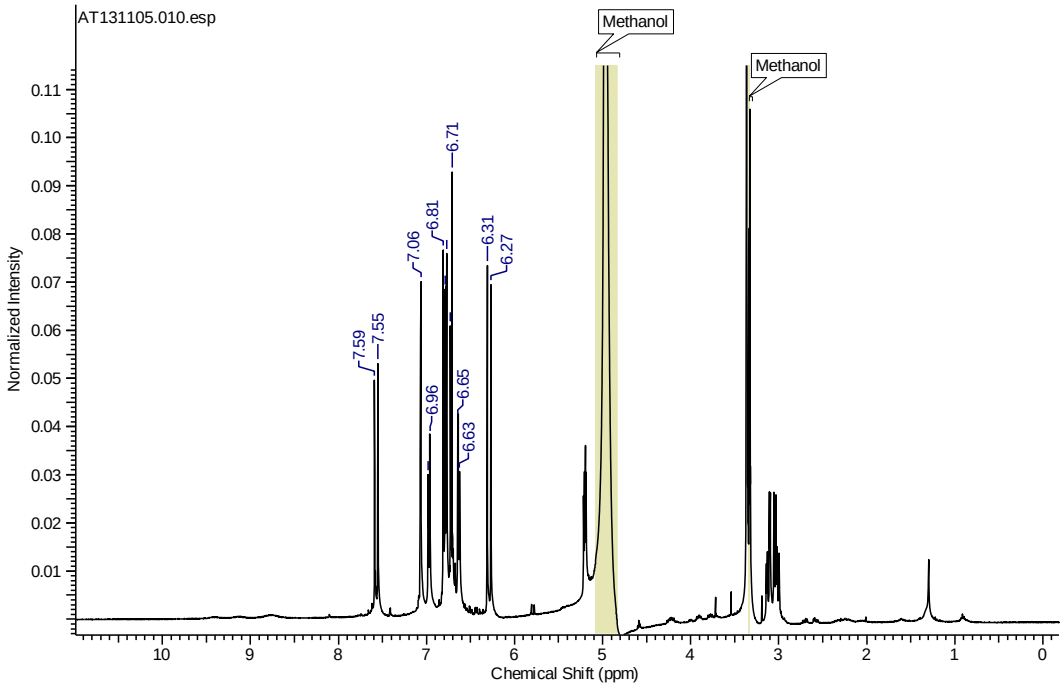


Figure 3.36 ^{1}H NMR spectrum (600MHz, CD_3OD) of 3.

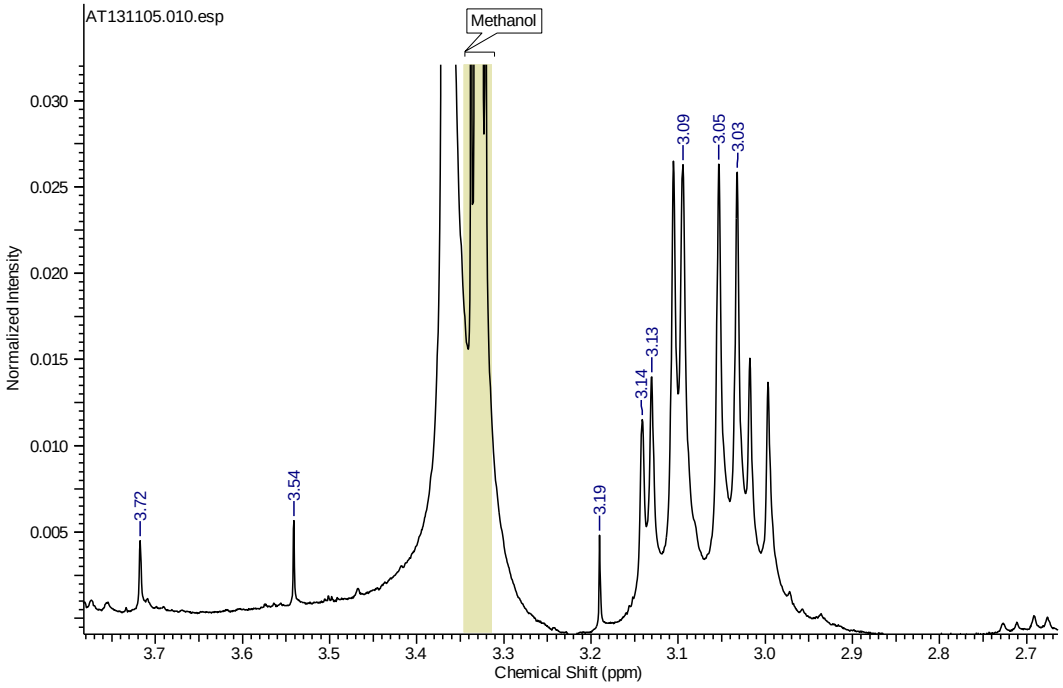


Figure 3.37 Expansion of ^{1}HNMR spectrum (600 MHz, CD_3OD) of 3.

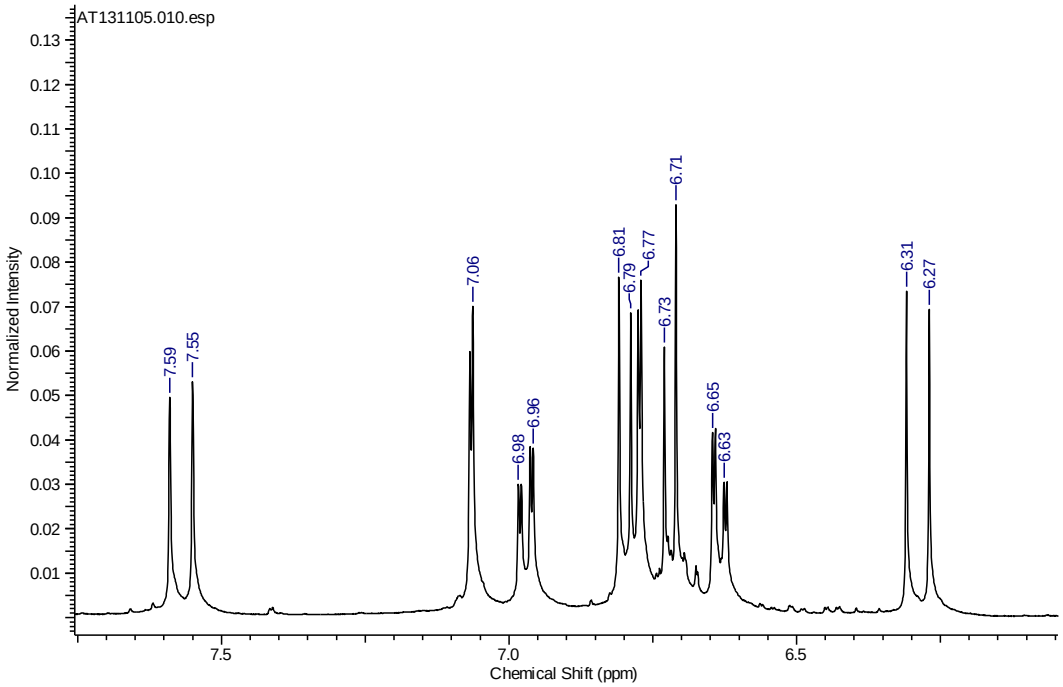


Figure 3.37 Continued.

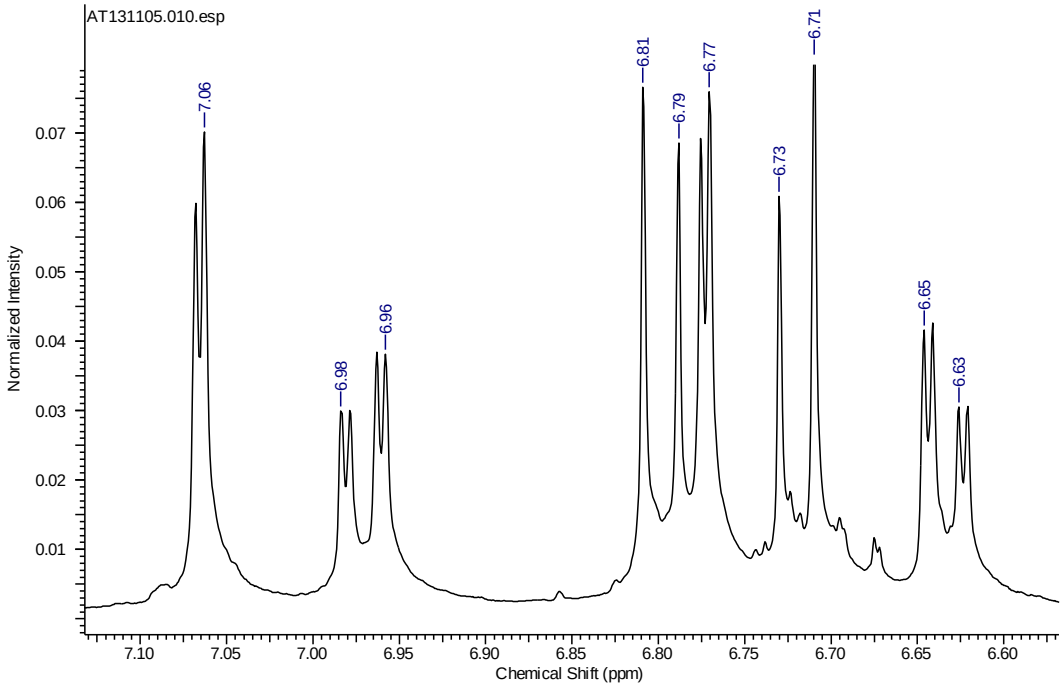


Figure 3.37 Continued.

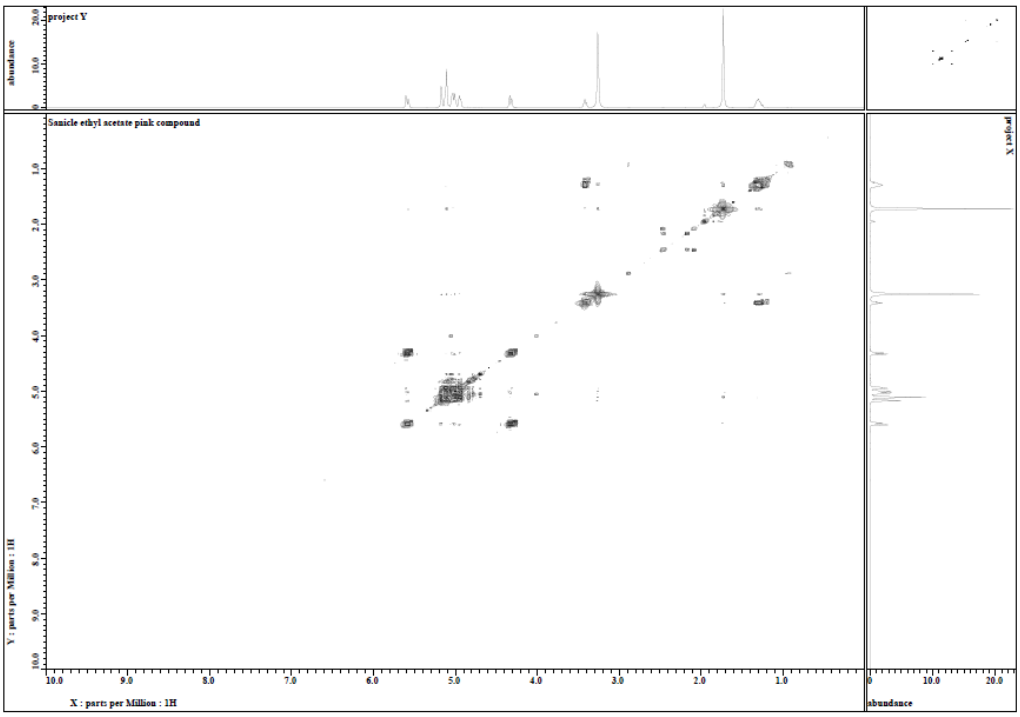


Figure 3.38 COSY- NMR spectrum (600 MHz, CD_3OD) of 3.

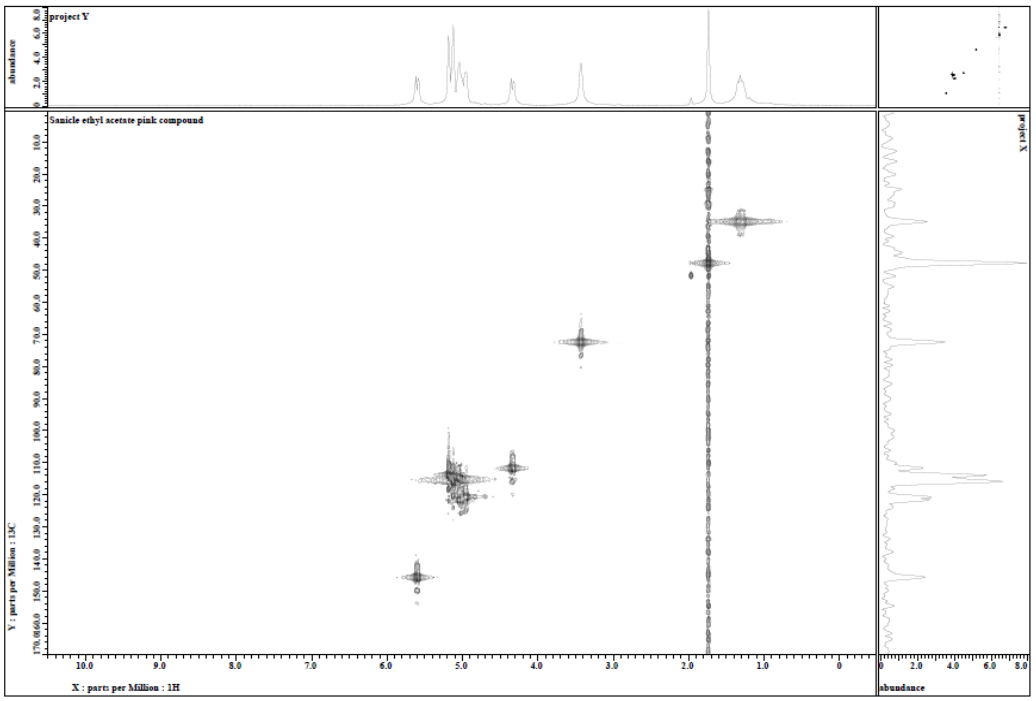


Figure 3.39 HMQC spectrum (600 MHz, CD_3OD) of 3.

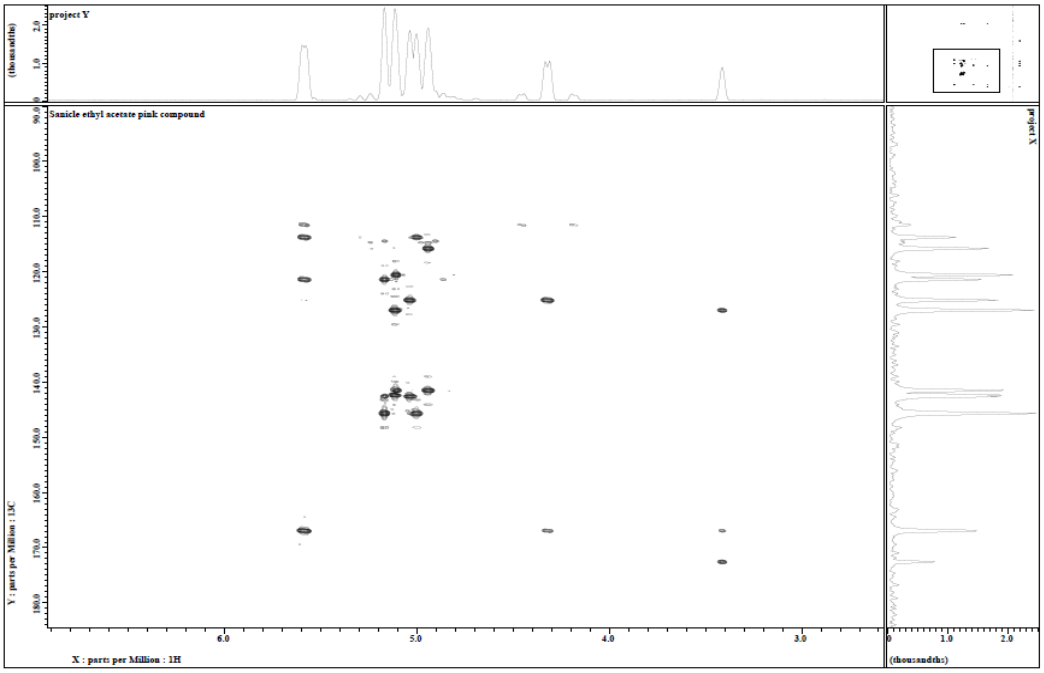


Figure 3.40 HMBC spectrum (600 MHz, CD_3OD) of 3.

3.4.6.2 Pharmacological actions of 3

Rosmarinic acid **3** is an active compound of many culinary plants (mint, meliss, prunella, sage, thyme, rosemary, orange, basil and sweet) (Karmokar *et al*., 2012), (Dubois *et al*., 2008).

Anusuya and Manoharan in (2011), studied the effect of **3** against 7,12-dimethylbenz(a) anthracene (DMBA) induced oral carcinogenesis. The results showed that **3** completely prevented the tumour formation and returned the status of biochemical and molecular markers to almost normal range in hamsters treated with DMBA. **3** (100 mg/kg body wt.) was found to stimulate detoxification enzymes, downregulate the expression of P53 and bcl-2 during DMBA induced oral carcinogenesis and improve the status of lipid peroxidation (Anusuya and Manoharan, 2011).

Compound **3** was found to prevent the development of cancer in preclinical studies. Therefore this study was aimed to evaluate the effect of **3** on development of intestinal adenomas. The results were compared with levels of **3** in the plasma and gastrointestinal tract. It is concluded that **3** inhibited the growth of Apc 10.1 cells derived from Apc ^{Min} mouse adenomas with IC_{50} value of 43 µm. The percentage of **3** in intestinal mucosa and in the plasma was found 38 nmol/g and 1.1 µm respectively (Karmokar *et al*., 2012).

The anti-inflammatory activity of rosmarinic acid was investigated in a murine model of respiratory allergy (asthma) by Ryan *et al* (2012). A/J mice model were used in thier study. Aspiratory allergy was induced by administration of Blomia tropicalis (BT) mite. The treatment was carried out using 2, 20 or 200 mg/kg of **3**. The results showed that treatment of animals with 100 mg/kg and 200 mg/kg of **3** led to a significant reduction in the numbers of leukocytes/eosinophils in bronchoalveolar lavage (BAL), like eosinophil peroxidas activity of BAL presence of mucus in respiratory

tract, histopathological changes in the lung and IL-4 in BAL (Costa *et al*., 2012).

The biological activity of **3** against (cis-diamminedichloroplatinum (II), Cisplatin (CP) induced nephrotoxicity was investigated by Rebert *et al*., (2014). Treatment with **3** considerably improved histopathological changes and the increase in serum creatinine and blood urea nitrogen (BUN) induced by CP. Administration of **3** significantly decreased hemeoxygenase (HO-1), cytochrome P450 ZE1, (CYPZE1) and renal 4-hydroxy nonenal (4-HNE) expression which were induced by CP. Furthermore, **3** showed antiapoptotic activity by decrease of P53, phosphorylated P53 and active caspase-3-expression in the kidneys (Domitrović *et al*., 2014).

The effect of **3** on atopic dermatitis (AD) was carried out by Jongsung *et al* (2008). In this study 7 men and 14 women aged between 5 to 28 years suffering from AD were involved. Emulsion of **3** at concentration of (0.3%) was topically applied to the elbow flexures of AD patients twice a day. All individuals were evaluated for skin conditions before treatment at the first visit and then at 4 and 8 weeks after treatment. According to AD index results, the erythema on antecubital fossa was significantly reduced at 4 and 8 weeks ($P < 0.05$). Self-questionnaires on effect of **3** indicated that dryness, pruritus and general AD symptoms improved; this suggest potential of clinical use of 4 as therapeutic agent for AD. **3** and it's acetyl ester derivatives which isolated from *Rosmarinus officinalis* were used as anti-inflammatory (Lee *et al*., 2008).

The nitration of **3** produced 6`- nitro and 6`,6``-dinitrorosmarinic acids. Both derivatives were found to have HIV-1, integrase inhibitors and anti-viral activity in MT-4 cells with similar selectivity indexes (Dubois *et al*., 2008).

The effects of **3** on a cultured neuronal cell line, SH-SY5Y *in vitro* and experimental ischemic diabetic stroke *in vivo* was evaluated by *Haiyun etal* (2013). **3** was incubated with tumour necrosis factor- α (TNF-α) stimulated SH-SY5Y cell line *in vitro* and oxygen-glucose deprived (OGD)-diabetic rats were used for an in vivo experiment. The rats were subjected to middle cerebral artery occlusion (MACO) for 40 minutes followed by reperfusion for 23 h. The results revealed that **3** decreased the OGD-induced apoptosis and cytotoxicity. In addition, **3** produced a considerable neuroprotective activity in rats with ischemia and reperfusion (I/R) at doses higher than 50 mg/kg (Luan *et al*., 2013).

3.4.7 Isolation of saniculoside N 4.

Fraction 11 (section 3.10) was further purified on normal phase silica gel column (section 2.5.1.) to yield **4**, the column conditions are showed in Table 3.18. Normal phase silica gel 30 g (for thin layer chromatography particle size 12 µm, section 2.5.1) was suspended in 200 ml chloroform to make a paste. Thin layer of sand was place a in the column. The paste was gently poured without disrupting the layer of sand at the bottom of the column. The silica allowed to settle and form a compact mass making sure that the level of solvent i.e. chloroform is above the surface of silica then place another band of sand on the silica mass. Fraction number 11 (284 mg) was suspended in approximately 5 ml of methanol. The sample was gently poured in the prepared column, the side of column washed with 80:20 chloroform:methanol to make sure the entire sample gets to the silica.

Table 3.18 Conditions of silica gel column chromatography of fraction C2 (section 3.3.2).

Mobile phase	% of composition	Volume (ml)	Fractions (7 ml)
$CHCl_3$: MeOH	80:20	50	1
$CHCl_3$: MeOH	70:30	100	2-6
$CHCl_3$:MeOH	60:40	100	7-13
$CHCl_3$:MeOH	50:50	100	14-20 compound **4**
$CHCl_3$:MeOH	40:60	100	21-26 compound **4**
$CHCl_3$:MeOH	35:65	100	27-31
$CHCl_3$:MeOH	30:70	100	32-37
$CHCl_3$:MeOH	20:80	100	38-43
MeOH	100	100	44-49

Fractions (17-26) table (3.18) 16 mg contained **4**. Preparative TLC (silica gel F_{254} aluminium sheets 20x20 cm plates, chloroform:methanol:water, 60:40:10 as a developing solvent) was carried out to yield 4 mg of **4**.

3.4.7.1 Structure elucidation of 4

Compound **4** was isolated as a white crystal from the crude glycosides extract of *S. europaea.* TLC analysis (section 2.5.5) of **4** showed no UV activity at 254 nm and a green spot after vanillin/sulphuric acid spraying. The ES- mass spectrum of **4** figure 3.41 showed a molecular ion peak $[M-H]^-$ at m/z = 1099, suggesting a molecular weight of 1100. Another peak was observed at m/z = 968 $[1101\text{-arabinose}]^-$. Table 3.19 is shown comparison of FAB-MS data of saniculoside N with the ES- mass data of **4**.

Table 3.19 Comparison FAB-MS data of saniculoside N with the ES-mass data of 4.

FAB(-) mass data of saniculoside N		ES- mass data of 4	
m/z	**Fragments**	**m/s**	**Fragments**
1099	$[M-H]^-$	1101	$[M-H]^-$
967	$[1099\text{-arabinose}]^-$	968	$[1101\text{- arabinose}]^-$
805	$[968\text{-glucose}]^-$	Not observed	-
587	[805-glucuronic acid propyl ester]$^-$	Not observed	-

^{1}H NMR spectrum (600 MHz, C_5D_5N) of **4** Figure 3.42 showed characteristic features of saponin compounds; therefore a revision of saponin compounds which isolated from *S. europaea* was carried out in order to find any matched data. The results revealed that saniculoside N was isolated from *S. europaea.* (Arda et al., 1997) has a molecular weight of m/z 1100 the same as **4**. A comparison of ^{1}H NMR data (600 MHz, C_5D_5N) of **4** with both ^{1}H NMR data (200 MHz, CD_3OD) of saniculoside N Table 3.20 and ^{1}H NMR data (200 MHz, $CDCl_3$) of saniculagenin N Table 3.21 revealed that **4** is saniculoside N.

Table 3.20 Comparison of ^{1}H NMR data (200 MHz, CD_3OD) of saniculoside N with ^{1}H NMR data (600 MHz, C_5D_5N) of 4.

^{1}H NMR data (200 MHz, CD_3OD) of saniculoside N	^{1}H NMR data (600 MHz, C_5D_5N) of 4
5.48 (1H, d, *J* = 9 Hz, H-1 of glucose)	5.31 (1H, d, H-1 of glucose)
5.81 (1H, d, *J* = 10 Hz, H-1 of arabinose)	5.60 (1H, d, H-1 of arabinose)
4.94 (1H, d, *J* = 7.6 Hz, H-1 of glucuronic acid)	4.40 (1H, d, H-1 of glucuronic acid)
3.00-4.30 (CH_2O-CHO of sugars protons)	3.00-4.30 (CH_2O-CHO of sugars protons)
6.06 (1H, q, H-3` of angelic acid ester)	6.20 (1H, dd, H-3`)
1.92 (3H, d, *J* = 7Hz, H-4` of angelic acid ester)	1.81 (3H, d, H-4`)
1.87 (3H, s, H-5` of angelic acid ester)	1.50 (3H, s, H-5`)

Table 3.21 Comparison of ^{1}H NMR data (200 MHz, CD_3OD) of saniculagenin N with ^{1}H NMR data (600 MHz, C_5D_5N) of 4.

^{1}H NMR data of saniculagenin N (200 MHz, $CDCl_3$)	^{1}H NMR data of 4 (600 MHz, C_5D_5N)
0.79-1.34 (6H, s, H-23 & H-27)	0.79-1.30 (6H, s, H-23 & H-27)
1.00 (6H, s, H-29 & H-30)	1.08 (6H, s, H-29 & H-30)
1.72 (3H, s, H-5`)	1.50 (3H, s, H-5`)
1.83 (1H, bd, *J* = 7Hz, H-4`)	1.81 (1H, bd, H-4`)
3.22 (1H, dd, *J* = 6.0, H-3)	3.00 (1H, dd, H-3)
3.31 (1H, d, *J* = 12 Hz, H-28`)	3.30 (1H, d, H-28`)
3.54 (1H, d, *J* = 12 Hz, H-28)	3.40 (1H, d, H-28)
3.92 (1H, d, *J* = 10 Hz, H-22)	3.69 (1H, d, H-22)
4.04 (1H, bd, *J* = 4Hz, H-16)	3.90 (1H, bd, H-16)
4.31 (1H, bs, H-15)	4.10 (1H, bs, H-15)
5.37 (1H, bs, H-12)	5.30 (1H, bs, H-12)
5.40 (1H, d, *J* = 10 Hz, H-21)	5.45 (1H, bd, H-21)
6.12 (1H, q, J = 7.0, 1.5 Hz, H-3`)	6.20 (1H, q, H-3`)

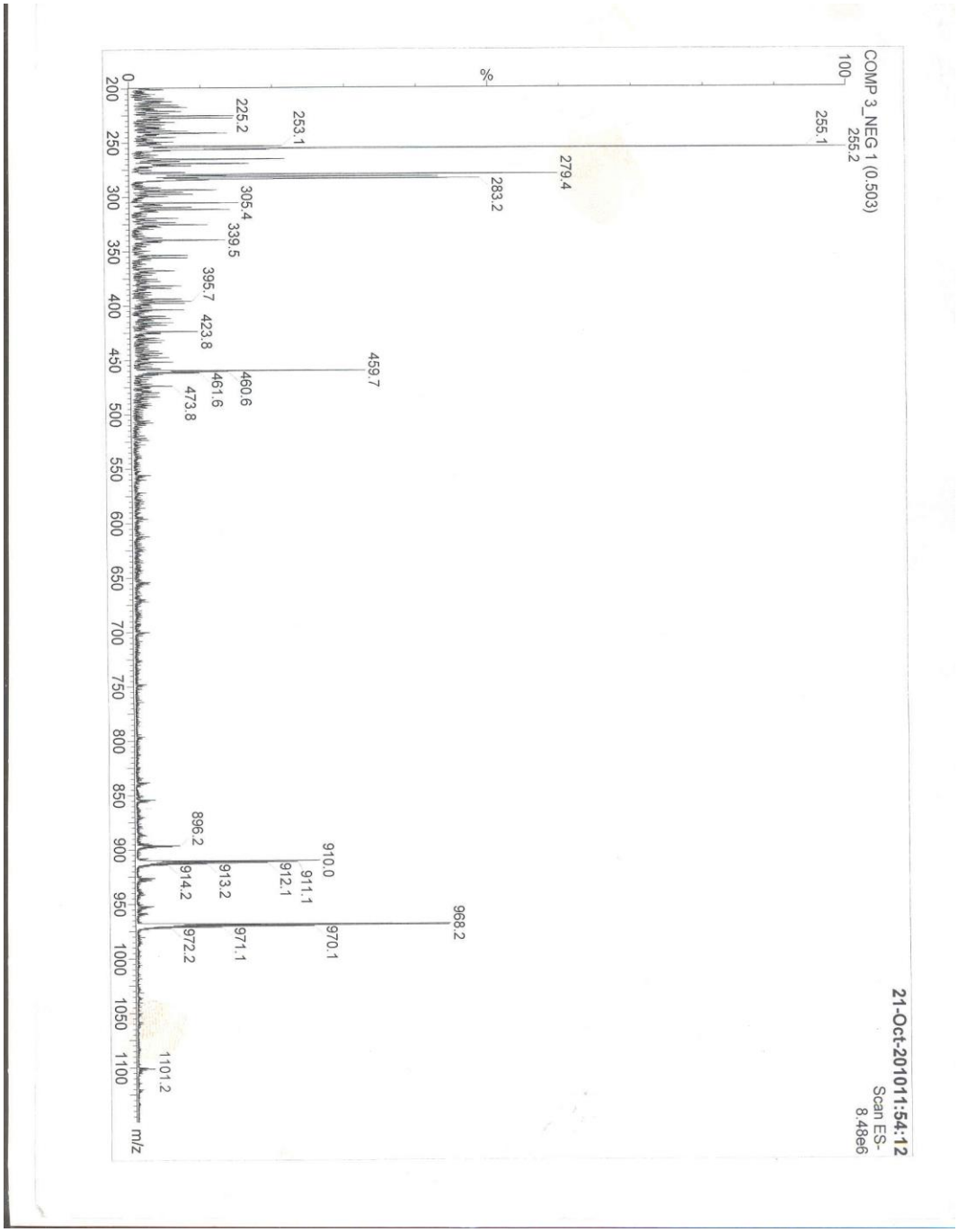


Figure 3.41 ES- mass spectrum of 4.

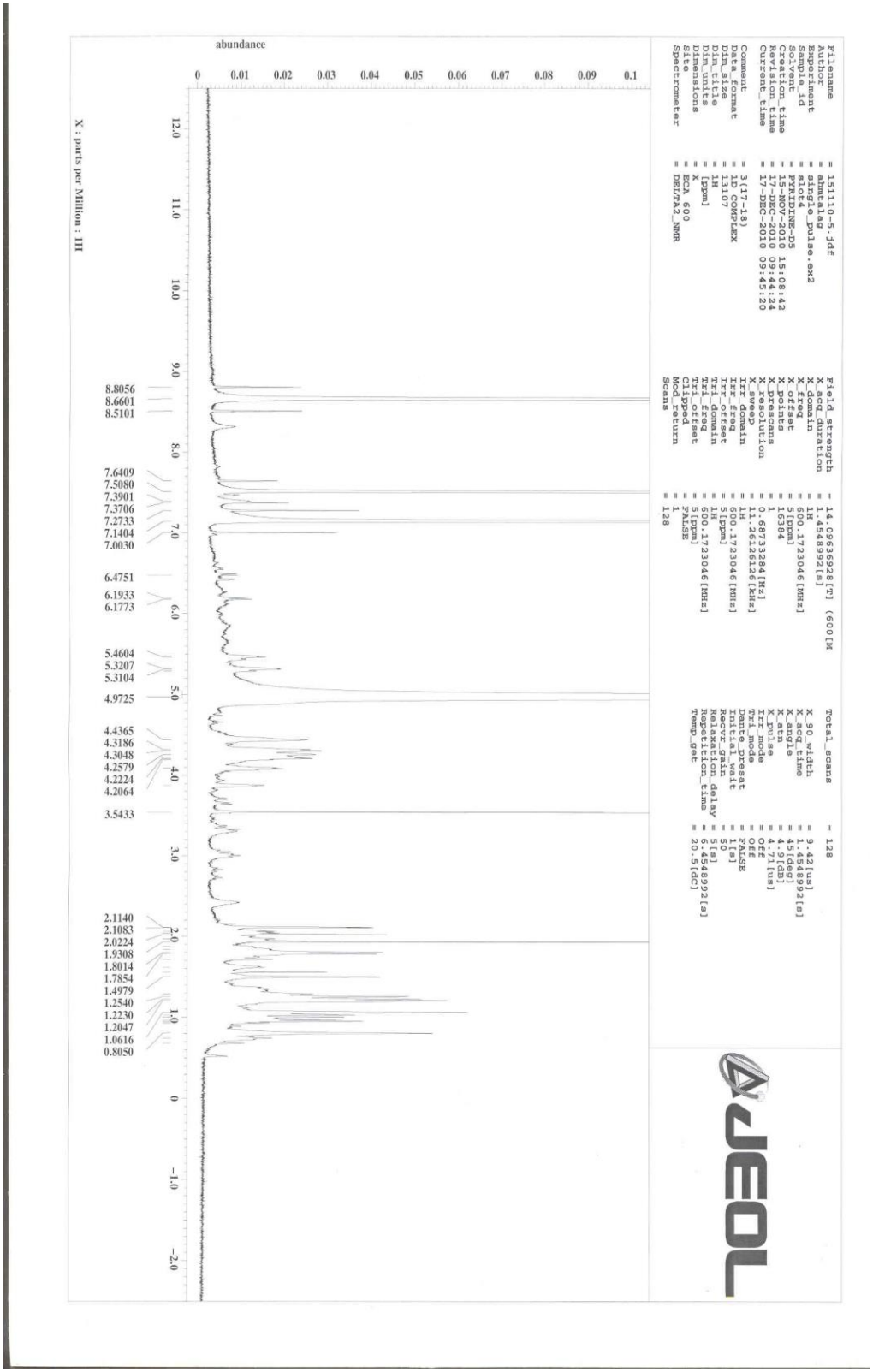


Figure 3.42 ^{1}H NMR (600MHz, C_5D_5N) spectrum of 4.

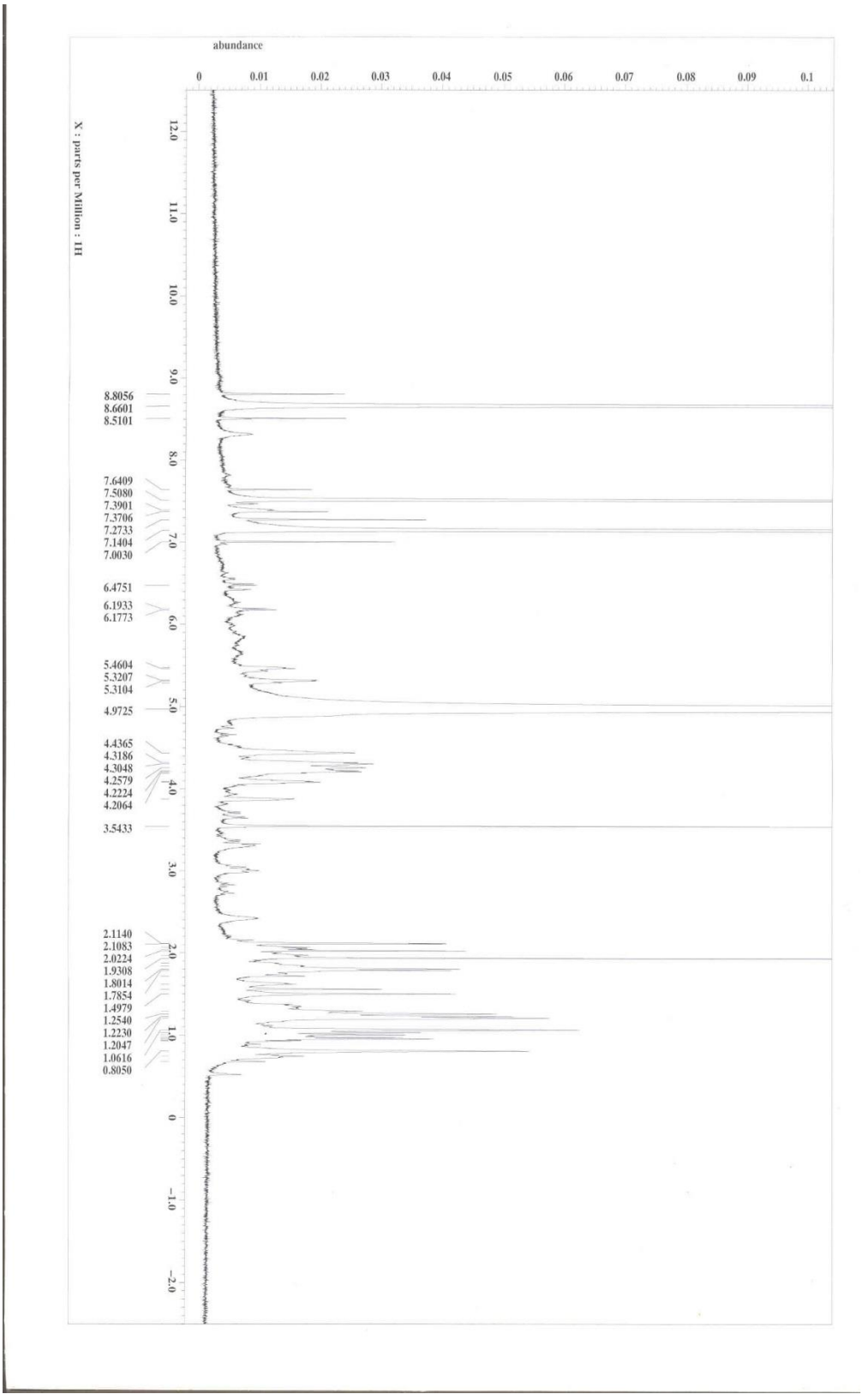


Figure 3.42 Continued

Taking in the account the different in the frequency and solvents in which ^{1}H NMR analysis of **4** and both saniculoside N and saniculagenin N were carried out, it can be concluded that **4** is saniculoside N. Furthermore, **4** was also isolated from *S. europaea* crude glycosides extract using HPLC technique (section2.3.6.3).

3.4.8 Isolation of sugar fractions (sucrose, glucose and fructose) 5

70 g of Sephadex LH20 were suspended in 450 ml of chloroform to make a suspension. The suspension was poured in the column. About 3.6 g of crude glycoside were dissolved in methanol (30 ml) than concentrated to (10 ml) of methanol extract. This extract was transferred to Sephadex LH20 column. 40 fractions of 25 ml each were collected. The chromatographic fractionation was monitored using silica gel 60 F_{254} aluminium sheets 20× 20 cm plates, chloroform:methanol:water, 60:40:10 as a developing solvent and vallinin sulphuric acid and anisaldehyde as a spray reagents.

Fraction number B2(44-67, 76 mg) section 3.3.2 contains two bands with a green colour on spraying, preparative TLC (silica gel 60 F_{254} aluminium sheets 20× 20 cm plates, chloroform:methanol:water, 60:40:10 as developing solvent) was carried out of the fraction, the two bands corresponding to the two compounds were collected. **5** was 16 mg was contained some impurity, so it was run again on a small TLC grad silica gel G6 column using 10 grams. The results are shown in Table 3.22. The quantity of other band was small so it discarded.

Table 3.22 Conditions of silica gel column chromatography of further purification of 5.

Mobile phase	% of composition	Volume (ml)	Fractions(7 ml)
$CHCl_3$:MeOH	80:20	50	1-7
$CHCl_3$:MeOH	70:30	100	8-21
$CHCl_3$:MeOH	60:40	100	22-26 compound **5**
$CHCl_3$:MeOH	50:50	100	27-33 compound **5**
$CHCl_3$:MeOH	40:60	100	34-39
$CHCl_3$:MeOH	30:70	100	40-43
$CHCl_3$:MeOH	20:80	100	44-47
Methanol	100	100	48

3.4.8.1 Structure elucidation of 5

Compound **5** was isolated as a white crystal from the crude glycosides extract of *S. europaea.* TLC analysis of **5** on silica gel $60F_{254}$ (section 2.5.1) showed no UV activity at 254 nm and a green spot after vanillin/sulphuric acid spraying (2.8.2). In the ES+ mass spectrum of **5** Figure 3.43 there is an evidence of glucose molecule m/z = 180 [m/z = 203 (M + Na), m/z = 360 (2M), m/z = 383 (2M + Na)], also there is an evidence of sucrose molecule m/z = 412 [m/z = 413 (M + 1), m/z = 334 (M - CH_2OH)].

Furthermore, because of sucrose was isolated from *Teucrium davaeanum* (section) therefore a comparison between the ES+ mass spectrum of **5** and accurate mass of sucrose **9** which isolated from *T. davaeanum* (Figure 4.46) was carried out. The following peaks were appears in both spectra (236.2, 245.1, 266, 301, 310, 334, 360.6, 361.4, 413 and 414).

The ^{13}C NMR spectrum of 5 Figure 3.44 (600 MHz, D_2O) showed 24 signals in which 20 were observed in the region between δ = 60 to δ = 80 which is characteristic to the sugars molecule. In addition to four signals in anomeric proton region, but the signal at 101 is for quaternary carbon

because it is not appear in the DEPT spectrum Figure 3.45 (600 MHz, D_2O). From the above data a total of 24 C signals of which three are anomeric protons are observed.

The ^1H NMR spectrum of **5** Figure 3.46 (600 MHz, D_2O) displayed signals at δ value in the region between δ = 3.20 to δ = 4.20 which is characteristic to sugars molecules, and it is a complex region indicated that the sample is composed from more than one sugars. The peaks in the downfield region of the ^1H NMR spectrum showed signals of three anomeric protons at δ 5.20, 4.64 and 4.19.

These data are in accordance with reference data from Gabriel *et al* (2013). The auteurs were identified the sugar fraction which isolated from *Apple Pomace*. It was composed from three sugars (glucose, sucrose and fructose) (Gabriel et al., 2013). By comparison of ^1H NMR data (600 MHz, D_2O) of **5** Figure 3.46 with the ^1H NMR data of sugar fraction of *Apple Pomace* (200 MHz, D_2O) Figure 3.47 it can be concluded that **5** is a mixture of three sugars (glucose, sucrose and fructose). TLC using standard sugar samples (sucrose, glucose and fructose) with **5** could be carried out to confirm the identity of the sample.

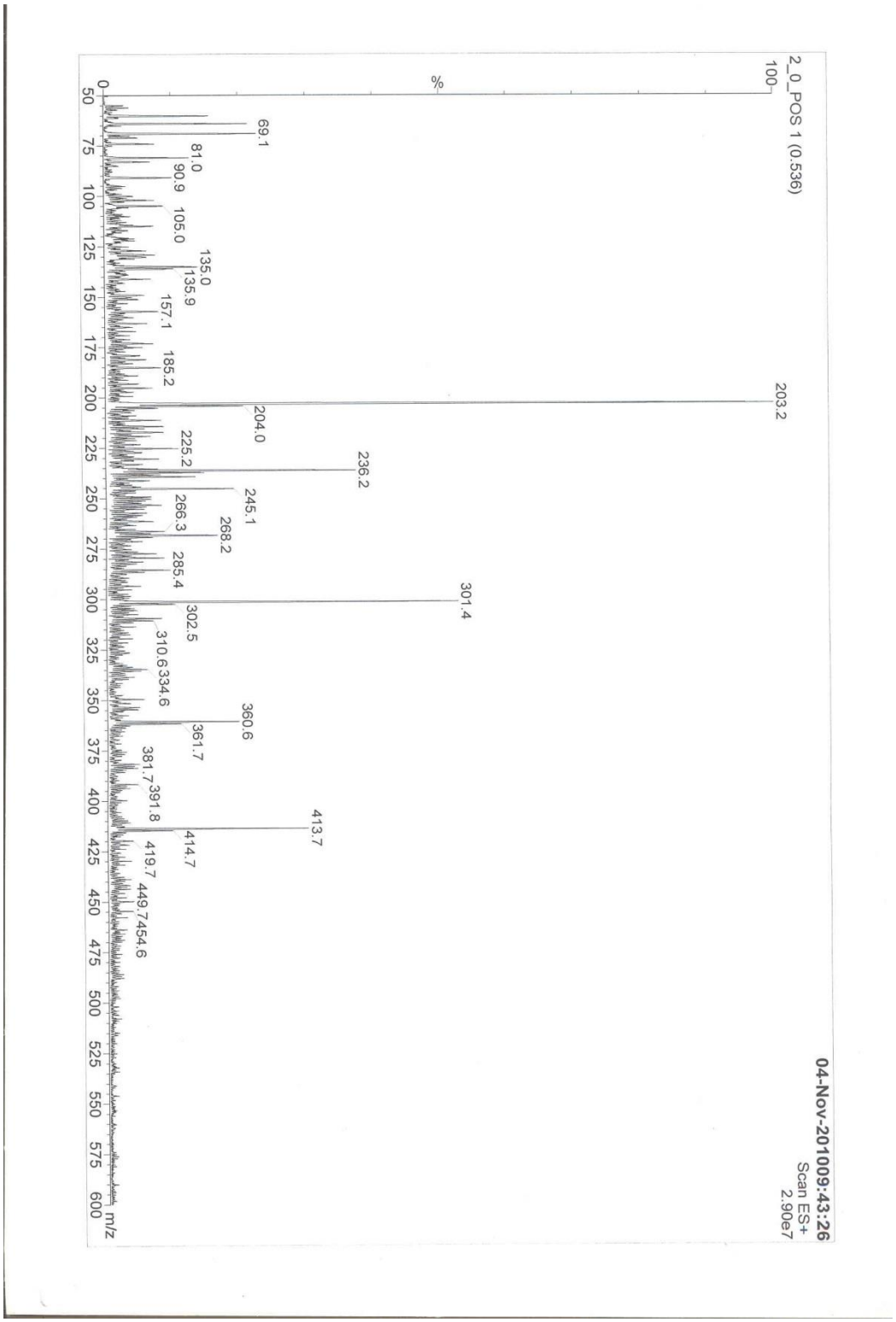


Figure 3.43 ES- mass spectrum of 5.

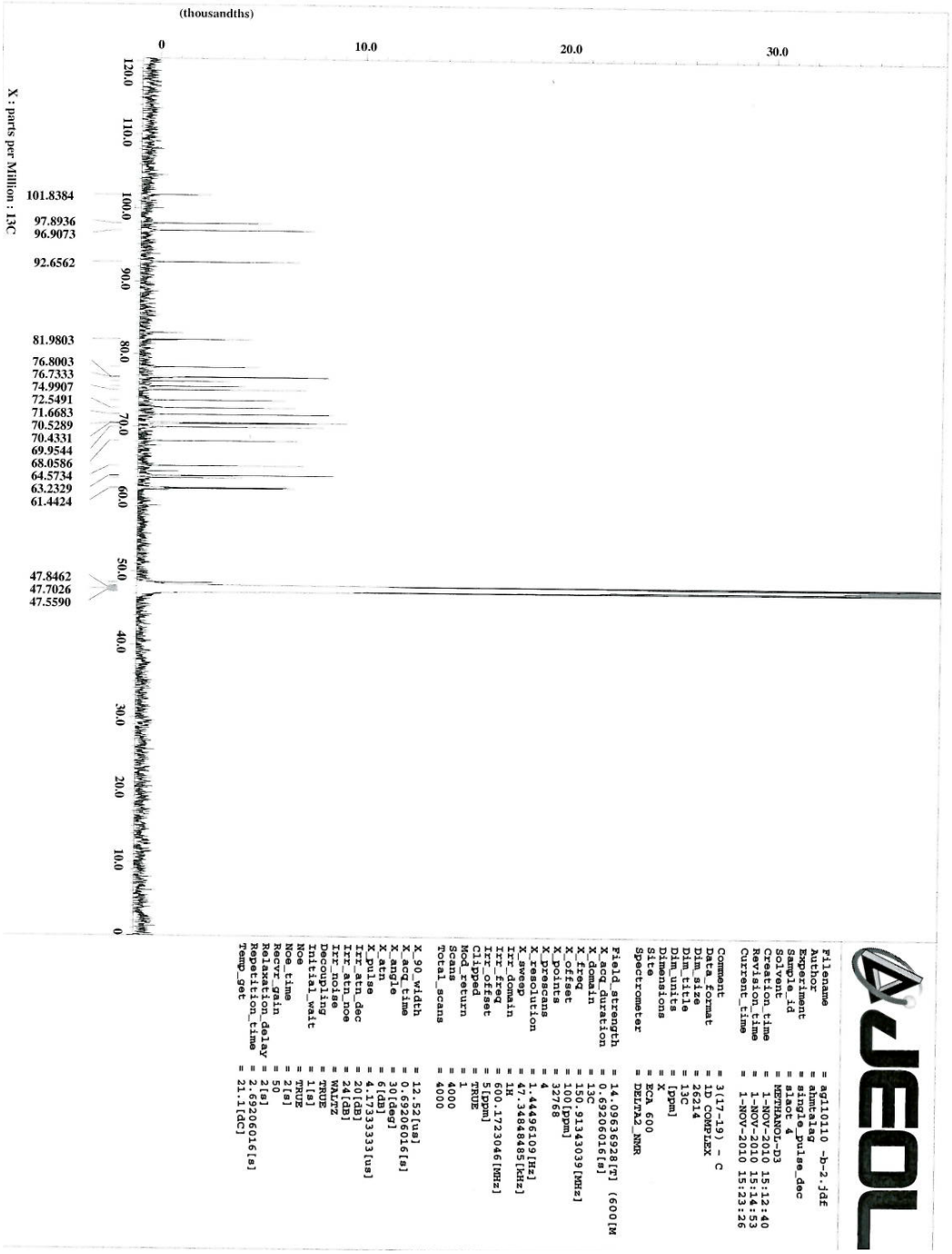


Figure 3.44 ^{13}CNMR spectrum (400 MHz, D_2O) of 5.

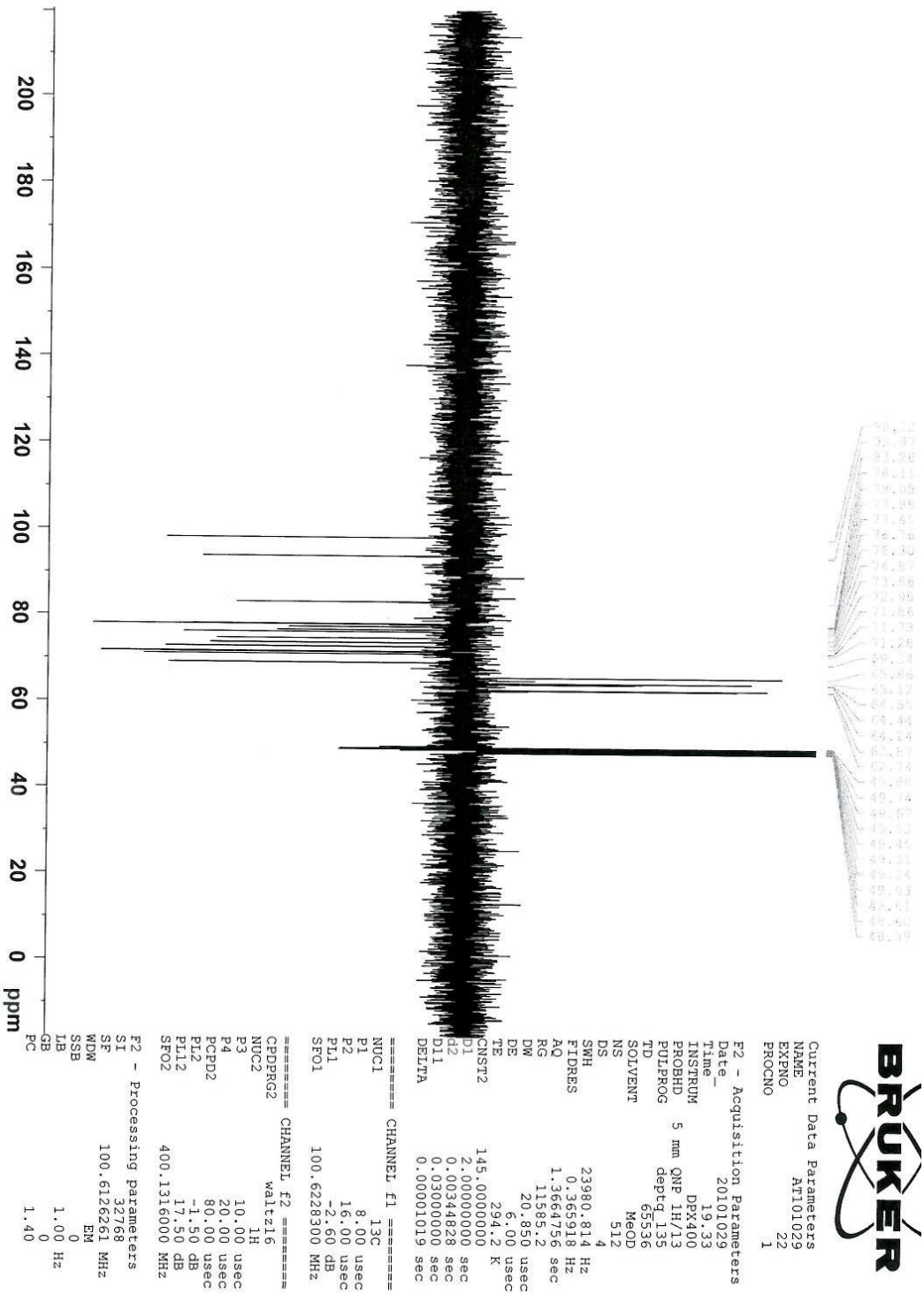


Figure 3.45 DEPT spectrum (400 MHz, D_2O) of 5.

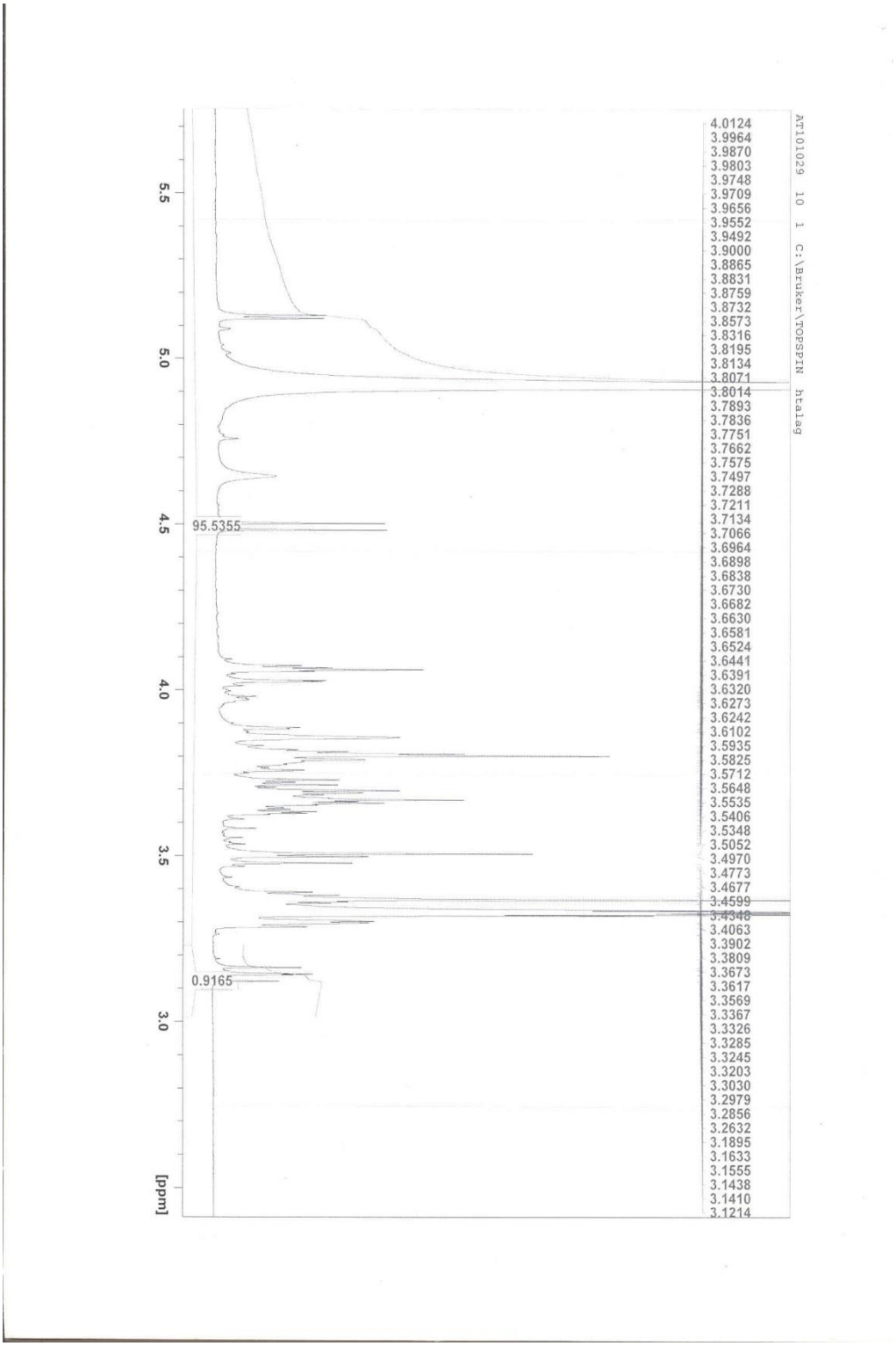


Figure 3.46 ^{1}HNMR spectrum (400 MHz, D_2O) of 5.

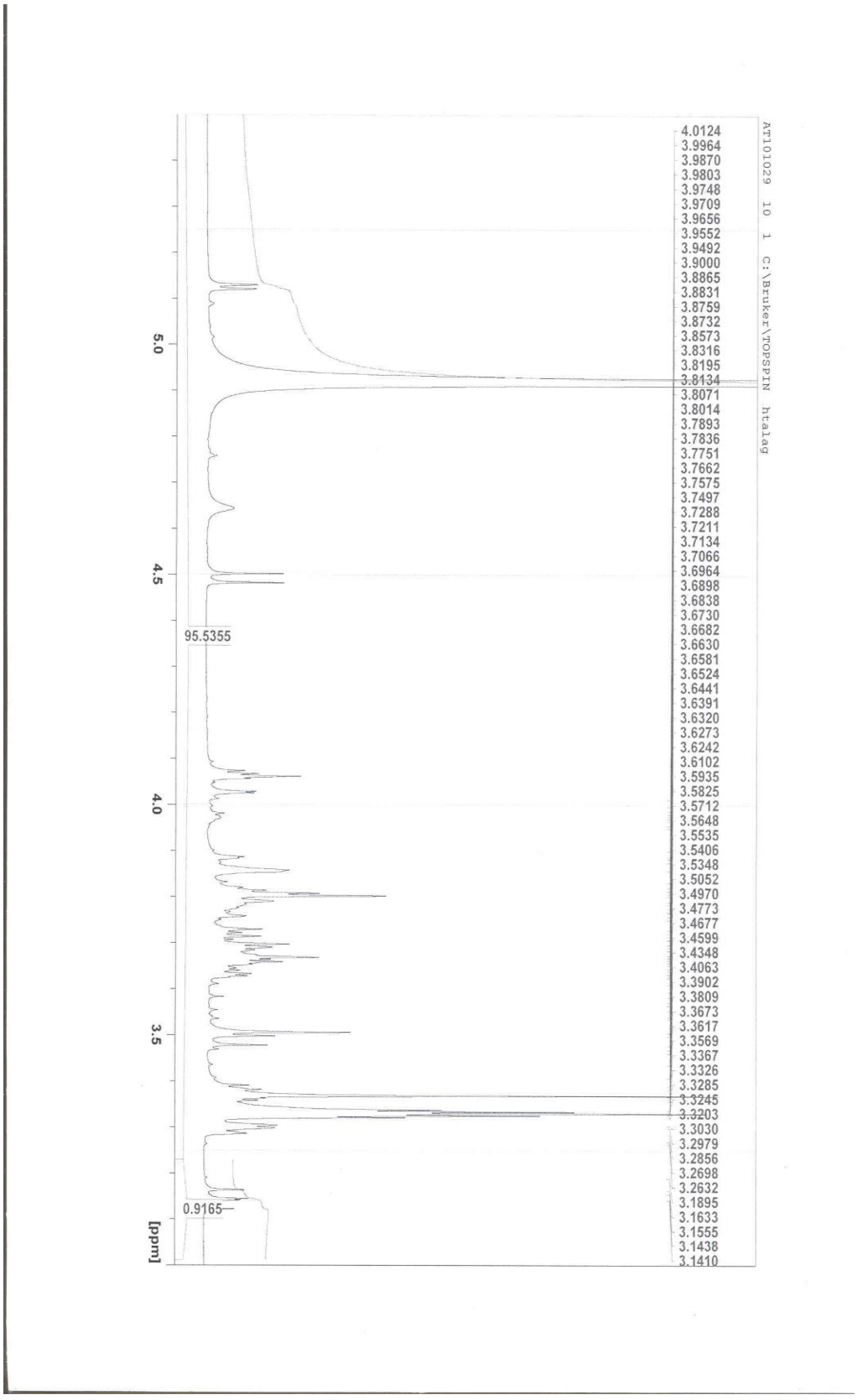


Figure 3.46 Continued.

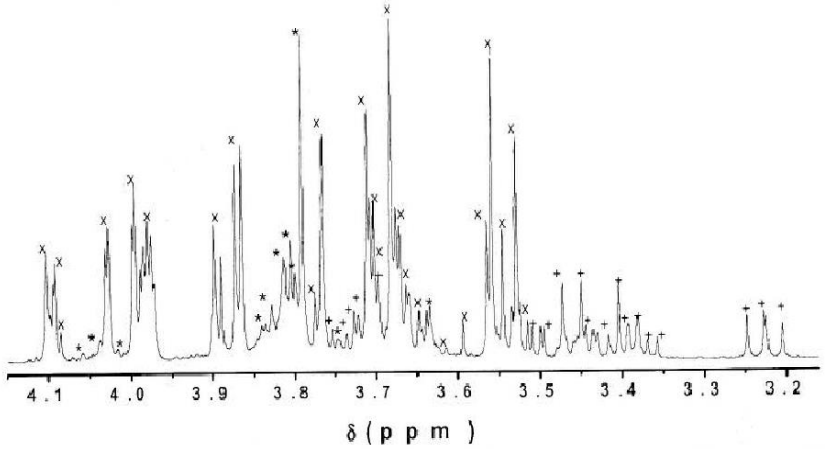


Figure 3.47 ^{1}HNMR spectrum (D_2O) of sugar mixture isolated from *Apple Poemes*.

3.5 Isolation of partial purified saponins mixture via HPLC system

In previous chapter open column chromatography, packed with either normal or reverse phase silica gel or sephdex LH-20 was used to purify the saponin glycosides from the crude glycosides extract of *S. europaea L*. This resulted in partial purification of saponin fractions A, B and C. Whilst preparative TLC was successful in purification of one saponin compound (**4)** the quantity was small and the method was time consuming. In this chapter, analytical HPLC was used to separate saponin compounds of a crude glycoside extract of *S. europaea L*, with the preparative HPLC technique being used to purify those compounds, to be used in further efficacy studies

3.5.1 Separation parameters of *S. europaea* glycosides

The saponin fractions of *S. europaea* (all samples prepared as 1 mg/ml) were separated by HPLC (section 2.7) using two types of columns; reverse phase silica gel C18 column and normal phase silica gel column (10 × 225 mm) injection loop 20 µl several mobile phases were used, flow rate varied between 1 and 7 ml/min.

Reverse phase silica gel column was used to separate the saponin fraction of *S. europaea* several mobile phases were tested on this column as illustrated in Table 3.23. The flow rate was also changed between 0.5 and 1 ml/min and the run time was between 30 and 60 minutes. The aim of variation in mobile phases and flow rate was to find out what parameters produce a best separation of saponin compounds.

Table 3.23 Composition of mobile phases tested for separation of saponins fraction B. on reverse phase HPLC column.

Mobile phase	% of composition	Flow rate ml/min.	Run time(min.)
MeOH:H_2O	90:10	1.0	30
MeOH:H_2O	70:30	1.0	60
MeOH:H_2O	50:50	1.0	30
MeOH:H_2O	45:55	1.0	60
MeOH:H_2O	40:60	1.0	30
MeOH:H_2O	35:65	1.0	30
MeOH:H_2O	35:65	0.5	30
H_2O	100	1.0	30
CH_3CN:H_2O	90:10	1.0	30
CH_3CN:H_2O	70:30	1.0	30
CH_3CN:H_2O	50:50	1.0	30
CH_3CN:H_2O	45:55	1.0	30
CH_3CN:H_2O	40:60	1.0	30
CH_3CN:H_2O	35:65	1.0	30
MeOH:H_2O:CH_3CN	50:50:50	1.0	60

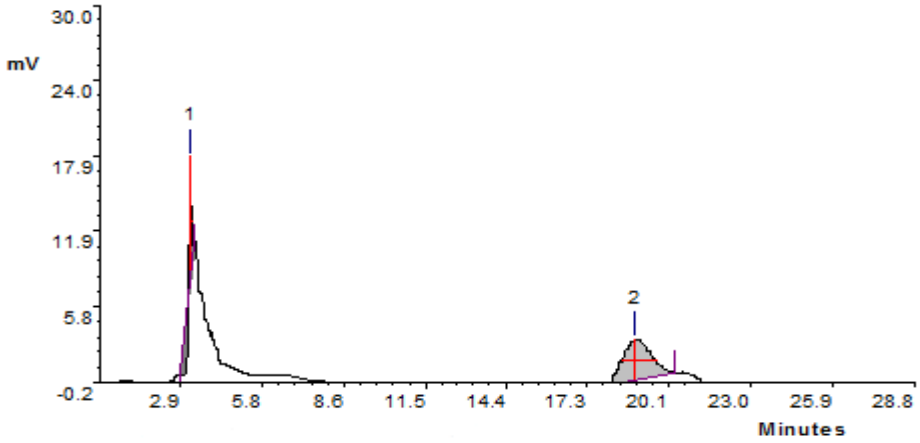


Figure 3.48 HPLC analysis of saponin fraction B, eluting $CH_3CN:H_2O$ 90:10.

It can be established from Figure 3.48 that a mobile phase compositions of $CH_3CN:H_2O$ 90:10, flow rate 1 ml/min, did not separate the saponin compounds from saponin fraction B (1mg/ml), as the targeted compounds eluted from the column as a single peak. TLC analysis of the saponin fraction B showed that the fraction comprised at least four compounds. Therefore a total of four peaks or more would be expected.

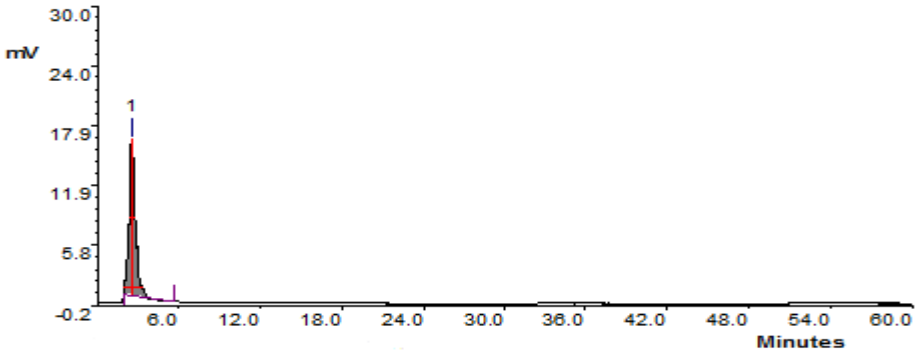


Figure 3.49 HPLC analysis of saponin fraction B, eluting $CH_3CN:H_2O$, 70:30.

Using of crude glycosides B (1mg/ml) silica gel C-18, mobile phase of $CH_3CN:H_2O$, 70:30, flow rate 1 ml/min, Figure 3.49 to separate the saponin compounds from saponin fraction B was not successful. Because the compounds still eluted from the column as a single peak. Therefore

the next experiment was to increase the polarity of mobile phase till 50:50, $CH_3CN:H_2O$.

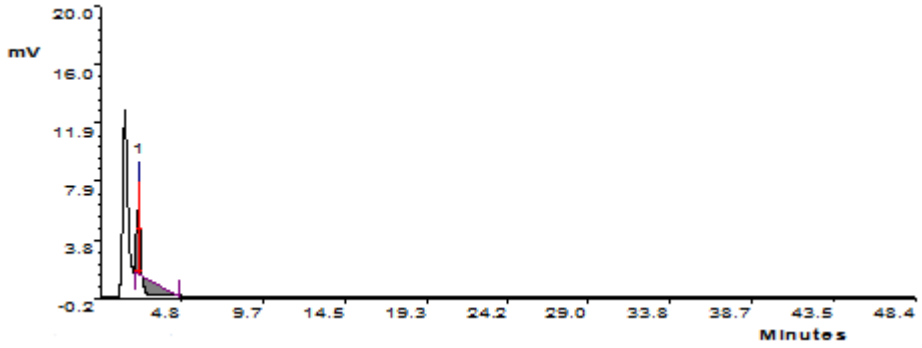


Figure 3.50 HPLC analysis of saponin fraction B, eluting $CH_3CN:H_2O$, 50:50.

The results of separation of the saponins fraction of *S. europaea* on reverse phase silica gel column connected to the analytical HPLC indicate that as amount of MeOH and CH_3CN in $MeOH:H_2O$ and $CH_3CN:H_2O$ decreased the separation is improved. The best chromatographs were resulted on using mobile phases compositions of 50:50 and 35:65 for both solvents $MeOH:H_2O$ and $CH_3CN:H_2O$.

Therefore, the next step was to use a new column (normal phase silica gel column 10 × 225 mm, loop 100 µl) a polar stationary phase which may produce a better separation to the saponin fraction B because the saponins will adsorb to the silica gel, so by increasing the polarity of mobile phase the less polar compounds will elute from the column first and the more polar compounds will elute at the end by increasing the polarity of mobile phase. Changes in both composition of mobile phase $CH_3CN:H_2O$, 50:50 and flow rate between 1, 2, 3, 5 and 7 ml/min were also suggested to improve the separation. Inclusion of a third mobile phase such as isopropanol and acetic acid was also recommended. The reason of changing in the percentage of compositions of mobile phase and the flow rate was to find out which parameters produce the best separation. Table 3.24 illustrates the compositions of mobile phases which

used to separate the saponins fraction B of *S. europaea* on normal phase silica gel column connected to HPLC technique.

At this step it can be concluded that two parameters could produce a better separation of saponins on normal phase silica gel column, increase the polarity of mobile phase and decrease the flow rate to 3 or 2 ml/min.

Table 3.24 Composition of mobile phases which tested for isolation of saponins fraction B on normal phase column

Mobile phase	**% of Composition**	**Flow rate ml/min**	**Notes**
CH_3CN:MeOH:Isprop-ol	35:60:5	5	Poor separation
CH_3CN:MeOH:Isprop-ol	35:55:10	5	Poor separation
CH_3CN:MeOH:Isprop-ol	35:55:10	3	Improved separation
CH_3CN:MeOH:Isprop-ol	35:60:5	3	Improved separation
CH_3CN:MeOH:Isprop-ol	35:60:5	7	Poor separation
CH_3CN:MeOH:Isprop-ol	40:55:5	5	Poor separation
CH_3CN:MeOH:Isprop-ol	35:60:5	2	Improved separation
$CHCl_3$:EtoAC:MeOH:H_2O	75:10:14:1	3	Poor separation
$CHCl_3$:EtoAC:MeOH:H_2O	75:10:14:1	5	Poor separation
$CHCl_3$:EtoAC:MeOH:H_2O	75:10:14:1	7	Poor separation
$CHCl_3$:MeOH: ACOOH	60:45:5	3	Poor separation
$CHCl_3$:MeOH: ACOOH	60:45:5	5	Poor separation
$CHCl_3$:MeOH: ACOOH	60:45:5	7	Poor separation
$CHCl_3$:MeOH:H_2O: ACOOH	60:45:4:1	3	Poor separation
$CHCl_3$:MeOH:H_2O: ACOOH	60:45:4:1	5	Poor separation
$CHCl_3$:MeOH:H_2O: ACOOH	60:45:4:1	7	Poor separation
$CHCl_3$:MeOH:H_2O	15:1:0	2	Poor eparation
$CHCl_3$:MeOH:H_2O	8:1:0.1	1.0	Poor separation
$CHCl_3$:MeOH:H_2O	60:40:10	5	Improved separation
$CHCl_3$:MeOH:H_2O	60:45:5	5	Improved separation
$CHCl_3$:MeOH:H_2O:Isopropanol	60:45:5: 5	5	Improved separation
$CHCl_3$:MeOH:H_2O:Isopropanol	60:45:5:10	5	Poor separation

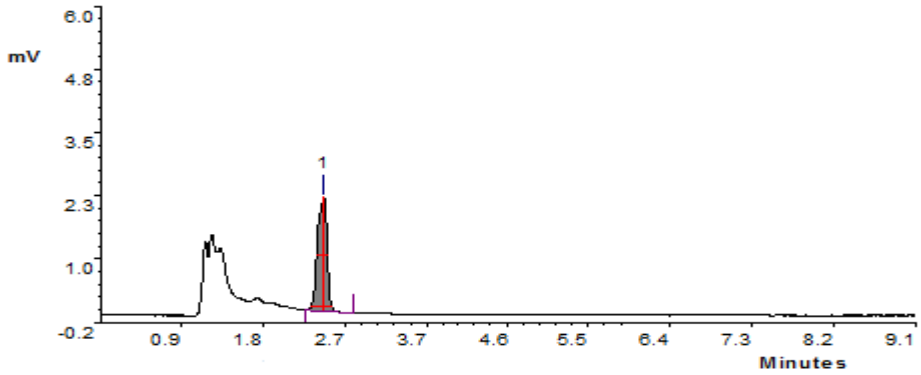


Figure 3.51 HPLC separation of saponin fraction B, eluting CH_3CN:H_2O:Isopropanol, 35:60:5.

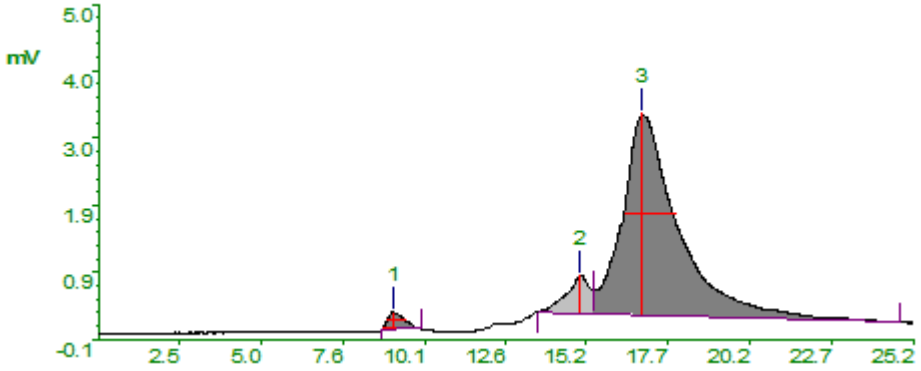


Figure 3.52 HPLC analysis of saponin fraction C, eluting $CHCl_3$:MeOH:H_2O, 60:45:5.

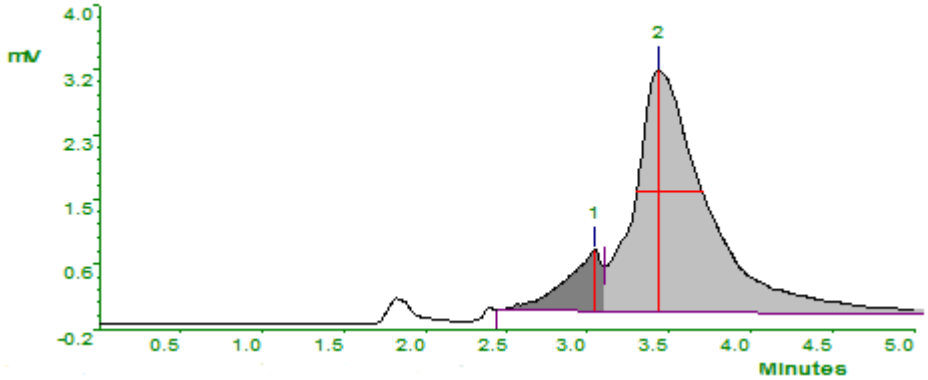


Figure 3.53 HPLC analysis of saponin fraction C eluting $CHCl_3$:MeOH:H_2O, 60:45:5.

The best two mobile phases for fractionation of saponins fraction of *S. europaea* on analytical HPLC column connected to normal phase silica gel column were CH_3CN:H_2O:Isopropanol, 35:60:5, flow rate 3 ml/min, and $CHCl_3$:MeOH:H_2O, 60:45:5, flow rate 1 ml/min because with decrease the flow rate, the saponins will have enough time to adsorb to silica gel, and the saponins will separate into several peaks instead of single peak. Therefore the less polar compounds will elute from the column first followed by more polar compounds.

The flow rate has a significant effect on purification of saponins fraction of *S. europa*ea on normal phase silica gel column, as the flow rate decreased as the peaks well separated on normal phase silica gel column figure (3.51), because the saponins separated into two distinguish peaks. Therefore the next step was fractionation of saponins using one of these two mobile phases.

3.5.2 Purification of saponins fraction via preparative HPLC system

Separation of saponins from *S. europaea* on analytical HPLC was best achieved using the mobile phase compositions of CH_3CN:H_2O:

Isopropanol, 35:60:5 at a flow rate 3 ml/min. A gradient system of CH_3CN:H_2O:0.1% formic acid was used as a mobile phase to do purification of the saponins fraction of *S. europaea* on a preparative HPLC system connected to C-18 silica gel column.

Four different gradient systems were used to purify the saponins fraction B (the purest sample) via preparative HPLC system connected to normal phase silica gel column. The sample was prepared by dissolving 6 mg in 1 ml distilled water (6mg/ml), the injection loop was 100 µl (injection of 0.1 mg each time), a luna C-18 column was used, the flow rate was 3 ml/min. Solvent A was composed from 5 % CH_3CN + 0.1 % formic acid, solvent B was composed from 95% CH_3CN + 0.1 % formic acid. The system was washed and equilibrated at the end of each run.

In the first method 100% solvent A was run for the first 10 minutes Table 3.25, followed by gradient to 40 % solvent B at 50 minutes, held for 50 minutes. The wash started at 65 minutes. In order to established at what UV wavelength region the saponin compounds absorb UV light, the preparative HPLC system (PDA) was set to 200 and 290 nm. The DAD spectral analysis showed that most of the polar compounds (hydrophilic peak) absorbed the UV light at three wavelengths 220 Figure 3.54, 290 Figure 3.55 and 330

Figure 3.56. While nonpolar compounds (hydrophobic) absorbed the UV light at one wavelength. Therefore, by using these methods there were two targets can be collected, a major peak eluted at 43 minutes and a number of nonpolar compounds eluted at 40 % CH_3CN.

Table 3.25 Gradient system applied in method 1 to purify saponins fraction B.

	Time	Flow	%A	%B	Curve
1	0.01	3.00	100.0	0.0	6
2	10.00	3.00	100.0	0.0	6
3	50.00	3.00	60.0	40.0	6
4	65.00	3.00	60.0	40.0	6
5	67.00	3.00	95.0	5.0	6
6	79.00	3.00	95.0	5.0	6
7	82.00	3.00	100.0	0.0	6
8	97.00	3.00	100.0	0.0	6

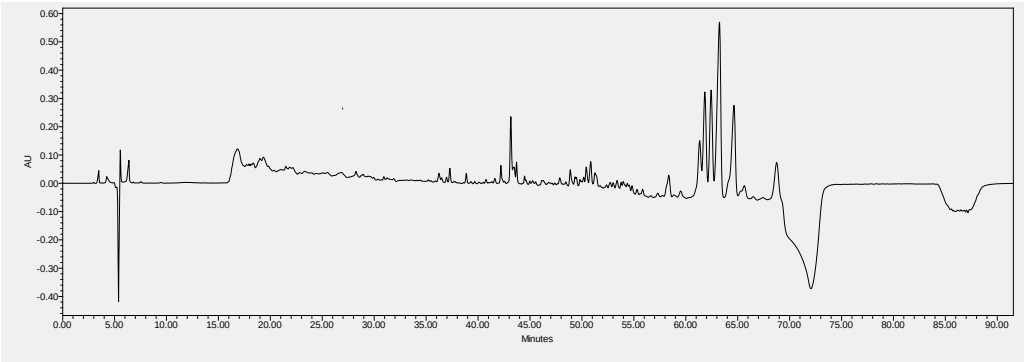


Figure 3.54 Compounds have absorption at 220 nm method 1.

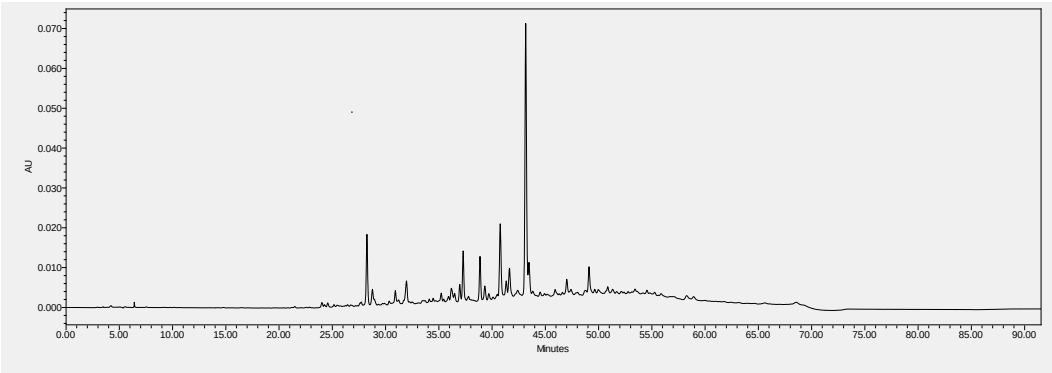


Figure 3.55 Compounds have absorption at 290 nm method 1.

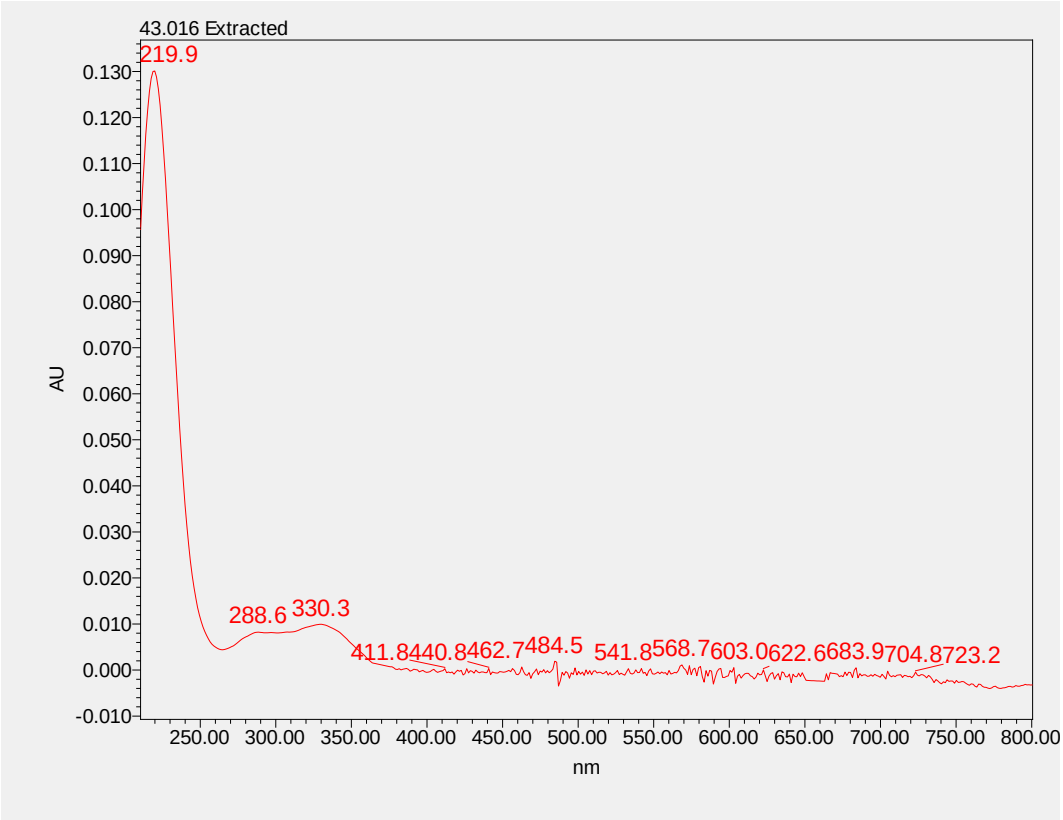


Figure 3.56 Compounds have absorption at 330 nm method 1.

The second method started at 10 % solvent B for the first 5 minutes Table 3.26, then gradients to 40 % solvent B at 35 minutes, held for 20 minutes then column wash was started at 55 minutes. There were two aims of the second method, reduced the run time and to improve the response from nonpolar compounds at a new wavelength 330 nm. The results showed that collection of the major polar peaks can be achieved by 16 minutes. Also, the separation of the non-polar compounds was improved. Figure 3.57 compounds which have a single maxima at 220 nm (method 2). Figure 3.58 compounds which have a single maxima at 290 nm (method 2). Figure 3.59 compounds which have a single maxima at 330 nm (method 2).

Table 3.26 Gradient system applied in method 2

	Time	Flow	%A	%B	Curve
1	0.01	3.00	90.0	10.0	6
2	5.00	3.00	90.0	10.0	6
3	35.00	3.00	60.0	40.0	6
4	55.00	3.00	60.0	40.0	6
5	57.00	3.00	95.0	5.0	6
6	69.00	3.00	95.0	5.0	6
7	72.00	3.00	90.0	10.0	6
8	87.00	3.00	90.0	10.0	6

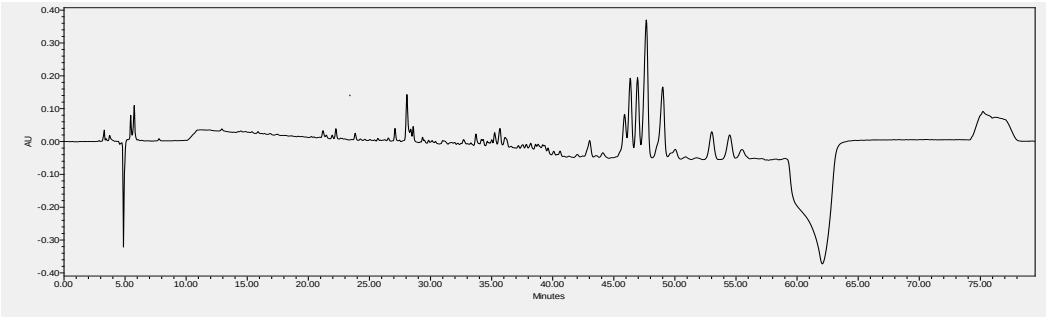


Figure 3.57 Compounds have absorption at 220 nm method 2.

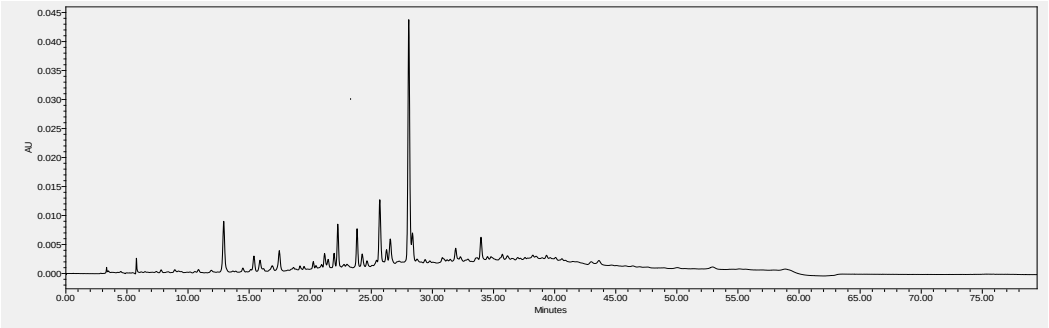


Figure 3.58 Compounds have absorption at 290 nm method 2.

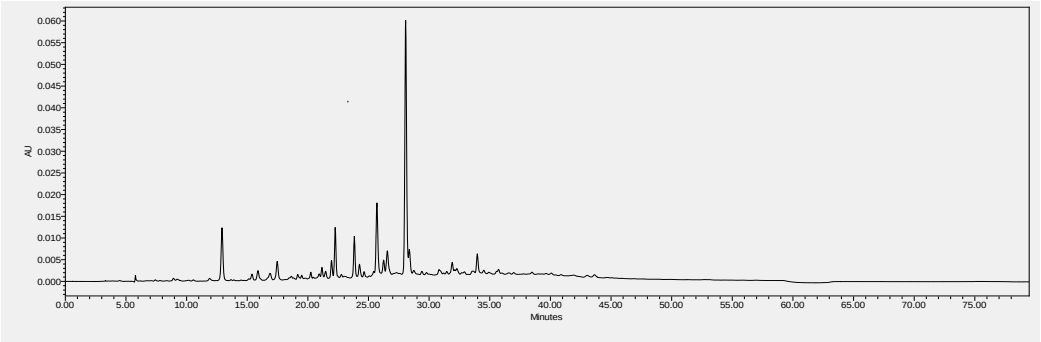


Figure 3.59 Compounds have absorption at 330 nm method 2.

The third method was started at 25 % solvent B for 5 minutes then gradient to 40 % B at 35 minutes followed by held for 20 minutes Table 3.27. The column wash was started at 55 minutes. The aim of method 3 was to attempt to resolve the major hydrophilic peak in the shortest time possible.

Figure 3.60 compounds which have a single maxima at 220 nm.

Figure 3.61 compounds which have a single maxima at 290 nm.

Figure 3.62 compounds which have a single maxima at 330 nm.

The results showed that at 330 nm all the early eluted polar compounds were combined into a single peak. Another peak was observed from approximately 10 minutes. The major peak was eluted at 13.5 minutes. While the later nonpolar compounds were eluted at 28 minutes. Table 3.28 and

Table 3.29 the gradient system applied in method three with reduce the run time. Table 3.30 is shown the gradient system applied in method three to purify the saponins fraction B with 25 % with 25 % solvent B for the first 35 minutes.

Table 3.27 Gradient system applied in method 3

	Time	Flow	%A	%B	Curve
1	0.01	3.00	75.0	25.0	6
2	5.00	3.00	75.0	25.0	6
3	17.00	3.00	69.0	31.0	6
4	18.50	6.00	5.0	95.0	6
5	24.50	6.00	5.0	95.0	6
6	26.00	6.00	75.0	25.0	6
7	30.00	6.00	75.0	25.0	6
8	32.00	3.00	75.0	25.0	6
9	34.00	3.00	75.0	25.0	6

Table 3.28 Gradient system applied in method 3 with reduce run time.

	Time	Flow	%A	%B	Curve
1	0.01	3.00	75.0	25.0	6
2	5.00	3.00	75.0	25.0	6
3	17.00	3.00	69.0	31.0	6
4	18.50	6.00	5.0	95.0	6
5	24.50	6.00	5.0	95.0	6
6	26.00	6.00	75.0	25.0	6
7	30.00	6.00	75.0	25.0	6
8	34.00	3.00	75.0	25.0	6

Table 3.29 Gradient system applied in method 3 with more reduce the run time.

	Time	Flow	%A	%B	Curve
1	0.01	3.00	75.0	25.0	6
2	5.00	3.00	75.0	25.0	6
3	17.00	3.00	60.0	31.0	6
4	20.00	3.00	5.0	95.0	6
5	32.00	3.00	5.0	95.0	6
6	34.00	3.00	75.0	25.0	6
7	46.00	3.00	75.0	25.0	6

Table 3.30 Gradient system applied in method 3 with 25 % solvent B for the first 35 minutes.

	Time	Flow	%A	%B	Curve
1	0.01	3.00	75.0	25.0	6
2	5.00	3.00	75.0	25.0	6
3	35.00	3.00	60.0	40.0	6
4	55.00	3.00	60.0	40.0	6
5	57.00	3.00	95.0	5.0	6
6	69.00	3.00	95.0	5.0	6
7	72.00	3.00	75.0	25.0	6
8	87.00	3.00	75.0	25.0	6

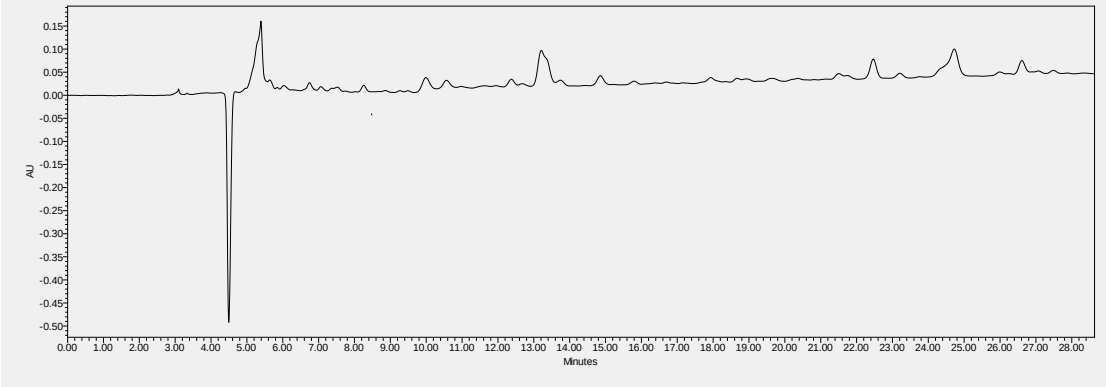


Figure 3.60 Compounds have absorption at 220 nm method 3.

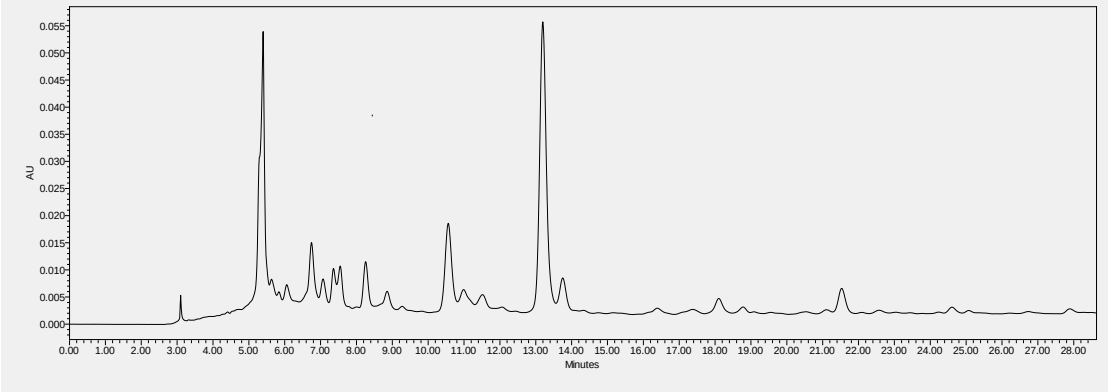


Figure 3.61 Compounds have absorption at 290 nm method 3.

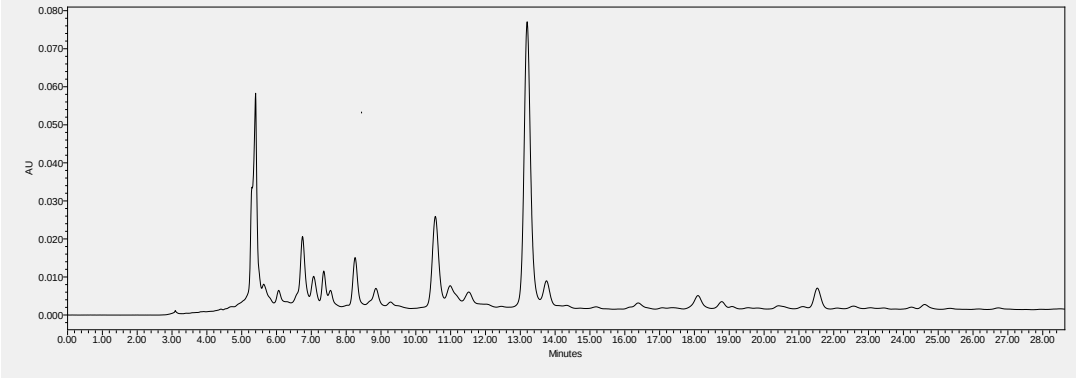


Figure 3.62 Compounds have absorption at 330 nm method 3.

The fourth method was held at 40 % solvent B Table 3.31 the purpose of this method was to significantly decrease the elution time for the major nonpolar compounds peaks which eluted at 18 minutes. The results showed that, the separation of both polar and nonpolar degraded compared to the previous method as illustrated in chromatogram 220 nm and 330 nm. Figure 3.63 compounds which have absorption at 220 nm. Figure 3.64 compounds which have absorption at 290 nm. Figure 3.65 compounds have absorption at 330 nm.

Table 3.31 Gradient system applied in method 4

	Time	Flow	%A	%B	Curve
1	0.01	3.00	60.0	40.0	6
2	55.00	3.00	60.0	40.0	6
3	57.00	3.00	95.0	5.0	6
4	69.00	3.00	95.0	5.0	6
5	72.00	3.00	60.0	40.0	6
6	87.00	3.00	60.0	40.0	6

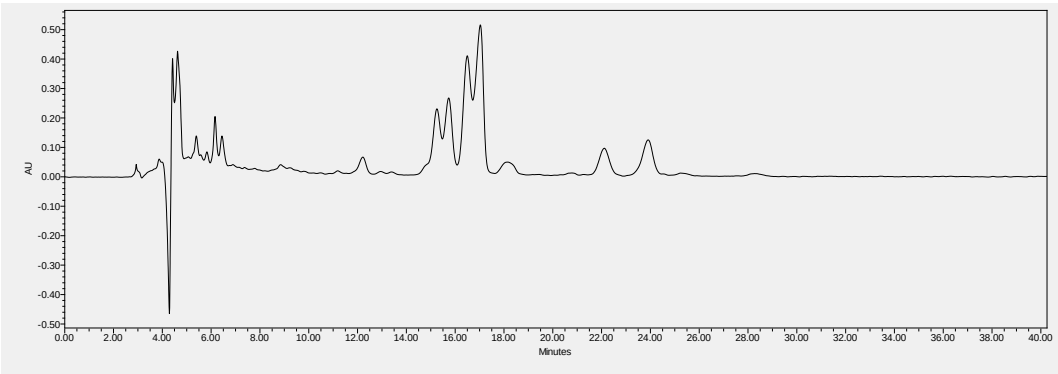


Figure 3.63 Compounds have absorption at 220 nm method 4.

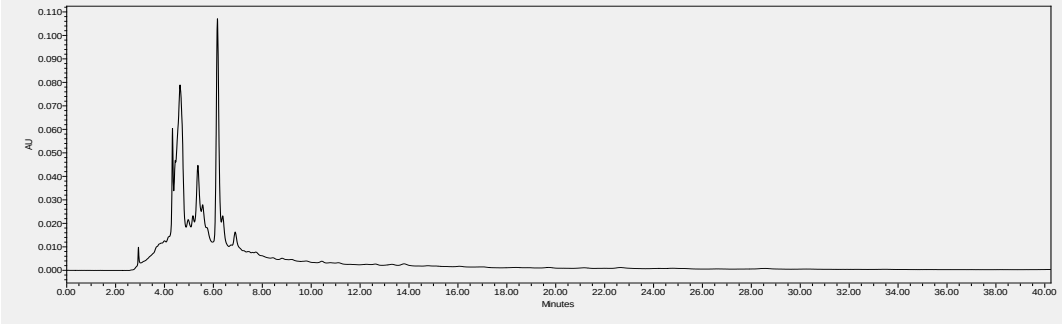


Figure 3.64 Compounds have absorption at 290 nm method 4.

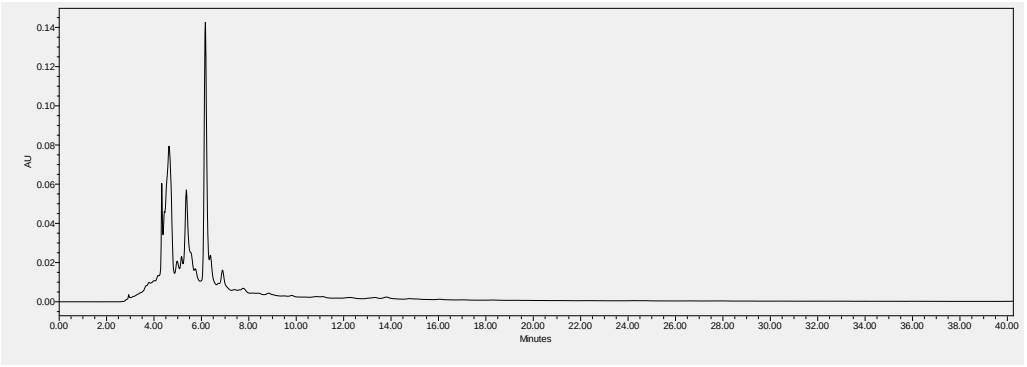


Figure 3.65 Compounds have absorption at 330 nm method 4.

The purification of a major polar compound peak could be achieved using method three, with total run time (including wash) of 30 minutes. Therefore, a total of 600 µg (injection loop 100 µl) of saponin fraction B is needed per run (0.1 mg per run). Since from the peak area it can be expected that the major peak is represented approximately 15-20 % of total eluted solids. Therefore, 10 run would yield approximately 1 mg (10 × 0.1 mg) pure compound (the major compound). Hence 20 run programme is recommended required 10 hours of practical work.

2.3.6.3 Purification of two glycoside compounds from saponins fraction B via preparative HPLC connected to normal phase silica gel column

The aim of the work was to use the method three (section 3.5.2) to purify the saponin fraction B of *S. europaea L.* The sample was prepared by dissolving 6 mg saponin fraction B in 1 ml distilled water, flow rate was 3 ml/min, injection loop 100 µl (lead to inject of 0.1 mg of physical sample each run), run time was 30 minutes. 20 runs were applied total practical work was 10 hours. At each run the follows peaks were collected in individual vials. The peaks at RT 5, 6.60, 7.40, 8.20, 10.40, 13 and 20 minutes.

The TLC results (silica gel 60 F_{254} coated aluminium sheets 20 × 20 cm) using chloroform:methanol:water, 60:40:10 as a mobile phase and vanillin sulphuric acid as a spray reagent, of collected fractions showed that, the fraction collected at 20 minutes was contained a pure compound (saniculaside N **4**) with a small impurity. The other fractions did not showed any constituents, which could be related to a very small quantity of these fractions.

the quantity of fraction eluted at 20 minutes was small (0.3 mg) and was sufficient only to do mass spectrometry and ^{1}HNMR measurements but other 1D and 2D NMR spectroscopy measurements and test the compound for treatments of wounds were not carried out because of the time shortage. Figure 3.66 shows mass spectrum of saniculoside N isolated from saponin fraction B via preparative HPLC. Figure 3.68 illustrates ^{1}H NMR spectrum of saniculoside N (400MHz, CD_3OD) isolated from saponin fraction B via preparative HPLC.

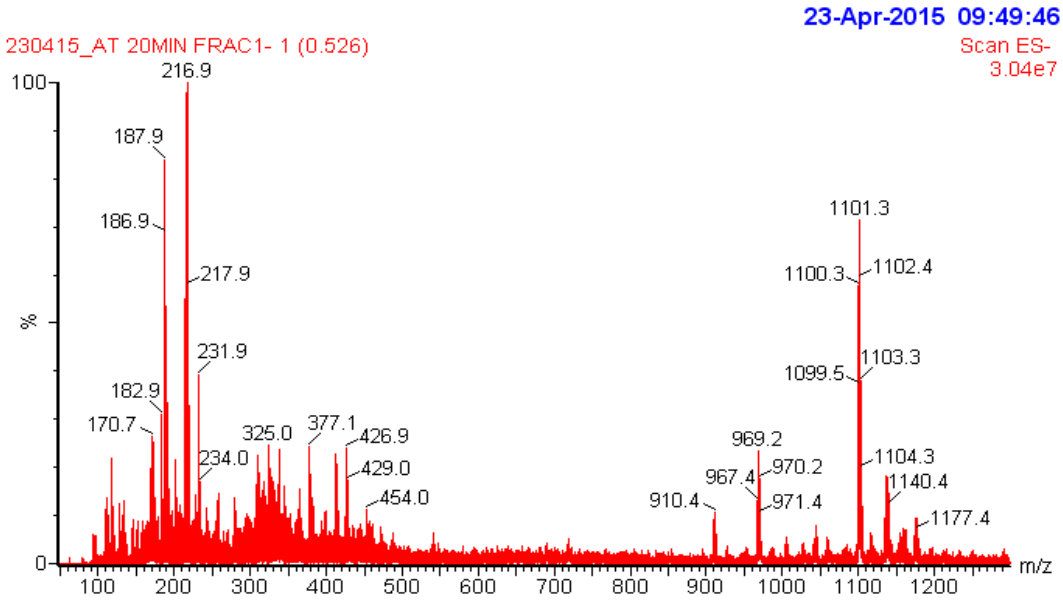


Figure 3.66 ES- mass spectrum of saniculoside N isolated from saponin fraction B via preparative HPLC.

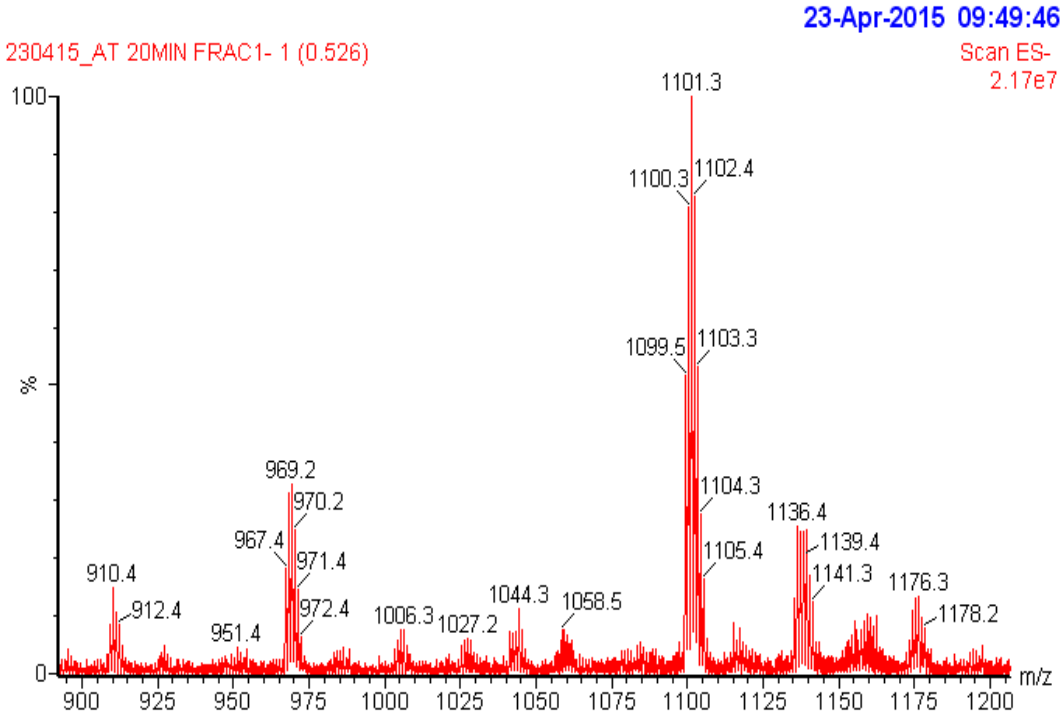


Figure 3.67 Expansion of ES- mass spectrum of saniculoside N isolated from saponin fraction B via preparative HPLC.

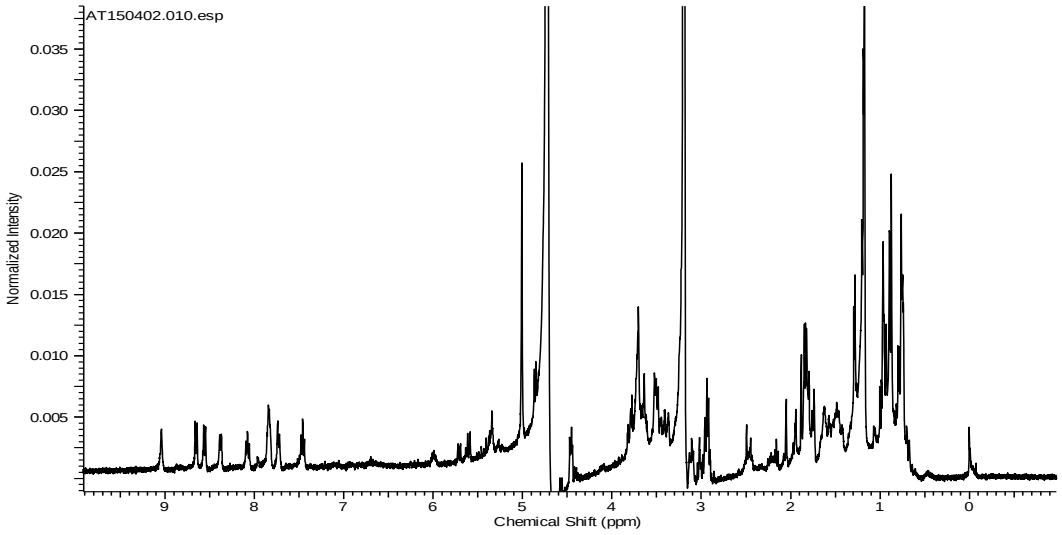


Figure 3.68 ^{1}H NMR spectrum (400MHz, CD_3OD) of saniculoside N isolated via PHPLC.

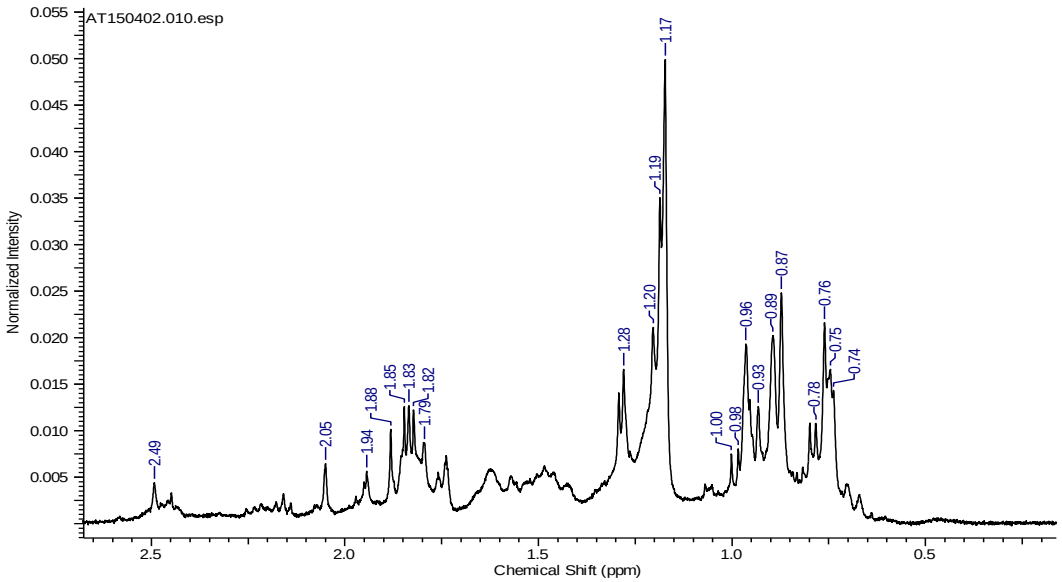


Figure 3.69 Expansion of ^{1}H NMR spectrum (600 MHz, CD_3OD) of saniculoaside N isolated via PHPLC.

The purification of saponins fraction B was finally successfully achieved via preparative HPLC connected to C-18 column. Six prominent peaks could be collected. A compound elute at 20 minutes (saniculoside N) was twice isolated from the saponins glycoside of *S. europaea* by (Arda et al., 1997) and in section (3.3.7) via preparative TLC. Further work needs to be carried out by repeating method 3 several times in order to collect a sufficient amount of compounds eluted at RT 5, 6.60, 7.40, 8.20, 10.40, 13 for ^{1}D & ^{2}D NMR measurements and test the identified compounds for treatment of wounds. Figure 3.70 Compounds isolated from 80% methanol extract of *S. europaea.*

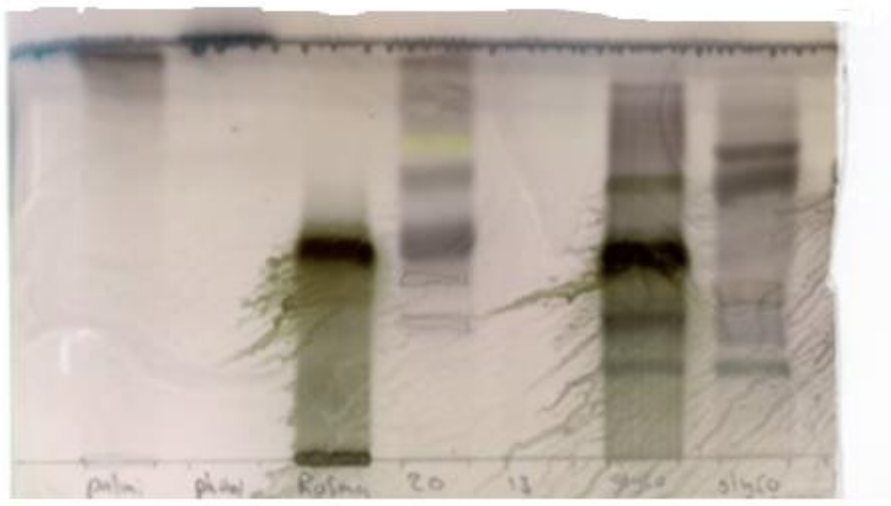


Figure 3.70 Compounds isolated from 80% methanol extract of *S. europaea*

4 Results and discussion, phytochemistry of *T. davaeanum*

4.1 Identification of the lipid constituents of *T. davaeanum*

Total lipid fraction of *T. davaeanum* was prepared as shown in section (2.4.1.). A total of 1 mg/ml from each sample (total fatty alcohol, total hydrocarbon and total fatty acid methyl ester) was dissolved in 1 ml dichloromethane and analysed on GC/MS instrument (section 2.6).

Table 4.1 Compounds identified in fatty alcohols ester of *T. davaeanum* via GC-MS.

No	Alcohols	Molecular formula	RT(min.)	Relative %
1	Heptacosane	$C_{27}H_{56}$	25.60	4.5
2	Octacosane	$C_{28}H_{58}$	27.26	35.4
3	Tetratetracontane	$C_{44}H_{90}$	28.48	51.0
			Total	90.9

Table 4.2 Compounds identified in hydrocarbons of *T. davaeanum* via GC-MS.

No	Hydrocarbons	Molecular formula	RT(min.)	Relative %
1	Caryophyllene	$C_{15}H_{24}$	12.48	0.96
2	Spathulenol	$C_{15}H_{24}O$	14.43	4.41
3	Caryophyllene oxide	$C_{15}H_{24}O$	14.52	3.66
4	Teu-Muurolol	$C_{15}H_{26}O$	15.16	3.34
5	α - Bisabololoxide B	$C_{15}H_{26}O_2$	15.30	11.12
6	α – Bisabolol	$C_{15}H_{26}O$	15.59	4.57
7	2-pentadecanone	$C_{18}H_{36}O$	17.25	1.66
8	Phytol	$C_{20}H_{40}O$	19.81	6.79
			Total	36.51

Table 4.3 Compounds identitied in fatty acids esters of *T. davaeanum* via GC-MS.

No	Fatty acids	Molecular formula	RT(min.)	Relative %
1	Decanoic acid methyl ester	$C_{11}H_{22}O_2$	11.11	0.35
2	14-octadecenal	$C_{18}H_{34}O$	12.2	1.07
3	Dimethyl phthalate	$C_{10}H_{10}O_5$	12.84	3.0
4	Dodecanoic acid methylester	$C_{13}H_{26}O_2$	13.67	8.27
5	Cyclopropaneoctanoic acid	$C_{20}H_{38}O_2$	13.93	1.03
6	Methyl tetradecanoate	$C_{15}H_{30}O_2$	15.95	7.12
8	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	18.04	14.56
				35.4

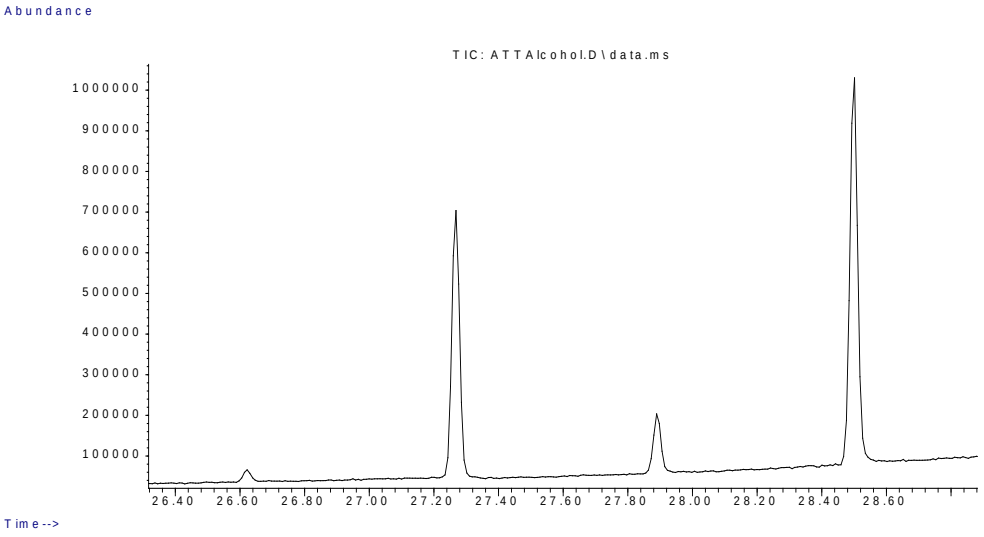


Figure 4.1 GC-MS analysis of fatty alcohol esters of *T. davaeanum*.

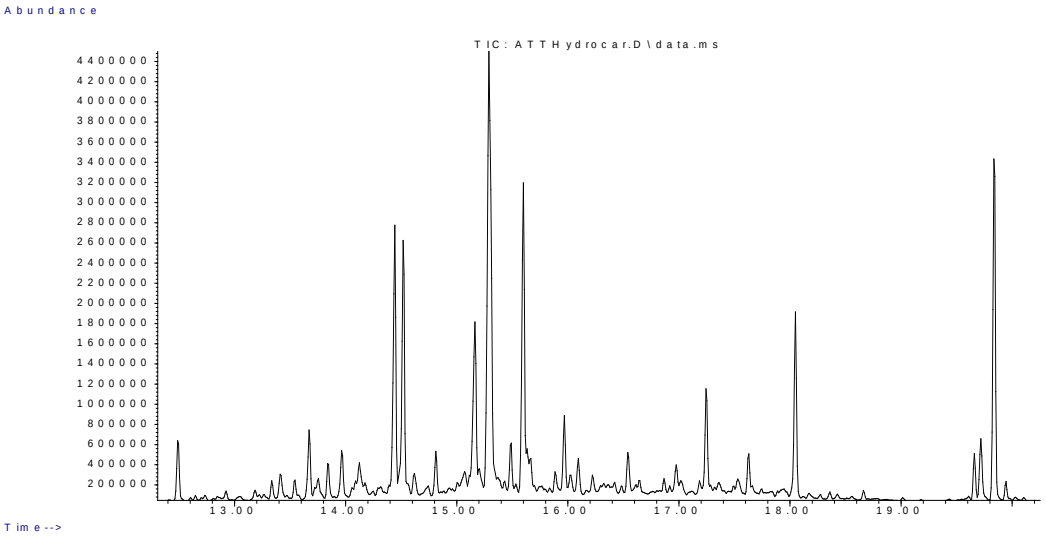


Figure 4.2 GC-MS analysis of hydrocarbon fraction of *T. davaeanum*.

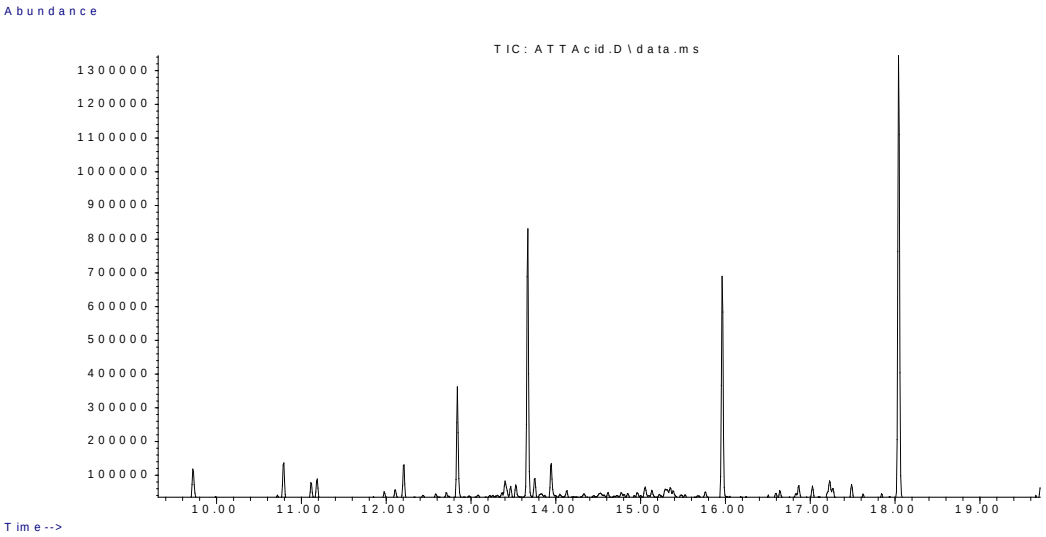


Figure 4.3 GC-MS analysis of fatty acid esters of *T. davaeanum*.

The results of GC-MS analysis of fatty alcohol methyl ester of *T. davaeanum* (Table 4.1 and Figure 4.1) showed the present of three hydrocarbons in which tetratetracontane the main one (51.0%), while the GC-MS analysis of hydrocarbon mixture (Table 4.2 and figure 4.2) revealed the present of phytol as main component (6.79%). Hexadecanoic acid methyl ester (palmitic acid) showed to be the major constituents of total fatty acid methyl ester of *T. davaeanum* (Table 4.3 and Figure 4.3). This compound mentioned in treatment of wound by (Victor R. Preedy et al., 2011) . Palmitic acid was isolated from the hexane extract of *S. europaea* (section 3.4.5) and also identified in GC-MS analysis of fatty acid methyl ester of *S. europaea* (section 3.1) therefore the wound healing activity of both species could be related to the present of this compound.

4.2 Preparation of crude glycosides extract of *T. davaeanum*

A total of 2.5 kg dried powdered plant material of *T. davaeanum* was extracted in a Soxhlet extractor (200 g each time) at 50 ^{o}C using 80 % methanol yielded 12 g of crude glycosides extract. Therefore each 1 g dried plant material of *T. davaeanum* contains 4.8 mg crude glycosides extract.

4.2.1 Fractionation of the crude glycosides of *T. davaeanum*

The crude glycosides extract of *Teucrium davaeanum* was fractionated by the same methodes that were used to do fractionation to the crude glycosides extract of *S. europaea* (section 2.4.1.2). A total of 5 g of crude glycosides of *T. davaeanum* were dissolved in 10 ml of methanol; 6 g of silica gel for thin layer chromatography (section 2.5.1) was added the mixture was ground until the liquid was absorbed. The mixture was fractionated on 200g of silica gel for thin layer chromatography (column 5 × 60 cm) under pressure as illustrated in Table 4.4.

Table 4.4 Fractionation of crude glycosides extract of *T. davaeanum.*

Mobile phase	Composition	Volume (ml)	Fr. number	Combined fractions (weight is not recorded)
$CHCl_3$:MeOH	100:0	200	1-5	
$CHCl_3$:MeOH	95:5	200	6-8	1-12
$CHCl_3$:MeOH	90:10	200	9-12	
$CHCl_3$:MeOH	80:20	200	13-15	13-15
$CHCl_3$:MeOH	70:30	200	16-18	17-18
$CHCl_3$:MeOH	60:40	200	19-21	19-20
$CHCl_3$:MeOH	50:50	200	22-24	23, 24, 25
$CHCl_3$:MeOH	40:60	200	25-27	26-27(compounds **7**&**8**)
$CHCl_3$:MeOH	30:70	200	28-30	28, 29-30
$CHCl_3$:MeOH	20:80	200	31-33	31-32
$CHCl_3$:MeOH	10:90	200	34-36	33-34 compound **6**, 35-36(Sucrose + Saponin)
$CHCl_3$:MeOH	0:100	400	37-42	37-38, 39-40 (Saponin comp.), 41-42

4.2.2 Isolation of compound 6

The results of TLC plates (section 2.5.5) of fractions (33-34, 256 mg) Table 4.4 showed they are composed of one compound and traces of impurity, therefore, 64 mg of these fractions were further purified using Sephadex LH20 (column dimensions 40 × 2 cm), methanol was used as eluting solvent, 19 fraction were collected subfractions 17 till 19 were in pure form (16 mg). Another three samples 64 mg each of fractions (33-34) were purified again on Sephadex LH20. The total pure quantity of **6** was 70 mg.

4.2.2.1 Structure elucidation of compound 6

Compound **6** was isolated as a brown powder from the crude glycosides extract of *Teucrium davaeanum*. TLC analysis of **6** (silica gel 60 F254, $CHCL_3$:MeOH:H_2O, 60: 40:10), vanillin sulphuric acid as spraying reagent) showed UV activity at 254 nm with a dark spot and a green spot after spraying R_f = 0.39, Figure (4.72). Compound **6** showed a positive colour with ferric chloride solution which indicates that it is a phenolic compound.

Compound **6** has the molecular formula $C_{35}H_{46}O_{19}$ (m/z = 770) as determined by an accurate mass measurement, the measured accurate mass was 788.2961 and the calculated accurate mass was 788.2972 corresponding to the molecular ion peak $[M + NH_4]^+$ Figure 4.4. The Accurate mass measurement was carried out at the EPSRC UK National Mass Spectrometry Facility at Swansea University. The ES- mass spectrum Figure 4.5 showed an $[M-H]^-$ ion peak at m/z 769 and the ES+ mass spectrum Figure 4.6 showed a peak at m/z 793 $[M+Na]^+$.

Table 4.5 shows the ^{1}H NMR, ^{13}CNMR and HMBC spectroscopic data of **6** (600 MHz,CD_3OD). The ^{13}C NMR spectrum of **6** (CD_3OD, 600 MHz) Figure 4.7 shows 35 signals, which are consistent with the molecular formula from the accurate mass, one of them is a carbonyl carbon because it appears at δ = 166.7 ppm. The DEPT spectrum of **6** Figure 4.8 shows 10 (-CH_2-) groups, 2 (CH_3-) and 20(-CH-) groups amounting 33 meaning that **6** has six quaternary carbons δ (145.5) C-3 and δ (143.3) C-4. Figure 4.9 shows an expansion of DEPT spectrum of **6**.

The ^{1}H NMR spectrum of **6** Figure 4.10 exhibited two trans olefinic protons at δ 6.26 (1H, *d*, *J* = 15.75 Hz, H-α`), 7.59 (1H, *d*, *J* = 15.87 Hz, H-β`) two ABX systems on 1,3,4-trisubstituted benzene rings δ 7.05 (1H, *d,* splitting pattern unclear, H-2````), 6.77 (1H, *d, J* = 8.19 Hz, H-5````) and 6.94 (1H, *d, J* = 8.06 Hz, H-6````); The signal at 6.67 (2H, *d, J* = 7.68 Hz, H-2,H-5) represent two protons H-2 and H-5; this was confirmed from the area of

the signal as it clearly represents 2 protons compared with other neighbouring signals which represent one proton. This consistent with the , HMBC spectrum since the signal at 6.67 shows correlations to 5 carbons, 4 appear to be 2 bond correlations δ 143.3 C-4, 120.0 C-6, 130.1 C-1, 145.5 C-3 and one carbon is a 3 bond correlation δ 35.4 C-β. From the compound structure H-5 is correlated to δ 143.3 C-4 , 120.0 C-6, 2 bond correlations, whereas H-2 is correlated to 130.1 C-1, 145.5 C-3 (2 bond correlations) and δ 35.4 C-β (three bond correlation). 6.67 (2H, *d, J* = 7.68 Hz, H-2 and H-5) and 6.55(1H, *d, J* = 7.81 Hz, H-6), also two aliphatic protons at δ 3.80 (2H, m, H-α) and 2.77 (2H, m, H-β). Figure 4.11 shows an expansion of ^{1}HNMR spectrum of **6** (600 MHz, CD_3OD). Figure 4.12 shows the COSY spectrum of **6**.

Furthermore, ^{1}H NMR spectrum showed the presence of three anomeric protons at δ 4.35(1H, d, H-1`), 5.16(1H, d, H-1``), 4.62(1H, d, H-1```) the attachments of three sugars were achieved by comprehensive studies of HMQC and HMBC spectra. Figure 4.13 shows the HMQC spectrum of **6**, Figure 4.14 illustrates an expansion of HMQC spectrum of **6**.

The outstanding HMBC cross peaks (Figure 4.15) from the anomeric proton of glucose δ 4.35(1H, d, H-1`) to the carbon atom C-α, δ (71.1) of the 5, 6-dihydroxy phenyl ethyl group allowed the connection of glucose anomeric carbon to that group. The caffeoyl unit was positioned at C-4` of glucose δ (69.5) based on the HMBC correlations between glucose proton at δ 4.97 (1H, m, H-4`) and carbonyl group at δ (166.7).

The first rhamnose unit is place d at C-3` of the glucose unit based on the HMBC correlations between C-3` δ 80.5 and the anomeric proton of the first rahmnose unit δ 5.16 (1``H, splitting pattern unclear). The second rhamnose unit is connected to C-6` of the glucose unit as shown from HMBC correlations between second rahmnose anomeric proton δ 4.62(1```H, splitting pattern unclear) and C-6` at δ 66.4 of glucose unit.

The splitting pattern of the two anomeric protons is unclear because both protons are shown as singlet at δ 5.17 and 4.62 for H1`` and H1``` respectively as shown in figure 4.10 , but theoretically doublets would be expected do you to the proton on adjacent carbons as showed from COSY data displayed in Table 4.5 Figure 4.12. This observation could be related to the stereochemistry of the two rhamnose anomeric protons.

The other support to this argument is that both anomeric protons of rhamnose units at δ 5.17, H1`` and δ 4.62, H1``` are correlated to the carbon atoms at C-2 and C-3 of the two rhamnose units i.e. H1`` δ 5.17 is correlated to C-2`` δ 69.33 and C-3`` at 71.04. While H1``` δ 4.26 is correlated to C-2``` δ 68.6 and C-3``` δ 71.20. Figure 4.16 expansion of HMBC spectrum of **6**, Figure 4.17 HMBC correlations of **6**.

The linkages between three sugars, caffeoyl moiety and phenylethyl group were confirmed by HMBC spectrum. Many but not all the signals are in agreement with those reported for the phenylethanoid glycoside isolated from *Teucrium chamaedrys* L named teucrioside Figure 4.18 (Bedir et al., 2003). Structure of **6** is shown in
Figure 4.19.

Table 4.6 shows ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) spectroscopic data comparison of **6** with 1H and 13C NMR (500 MHz, CD_3OD) of teucrioside isolated from *Teucrium chamaedrys L* Figure 4.18 (Bedir *et al*., 2003).

Table 4.5 ^{1}H NMR, ^{13}CNMR and HMBC data (600 MHz, CD_3OD) of 6.

No	δ_H (600 MHz, CD_3OD) of 6	δ_C (600 MHz, CD_3OD) of 6	HMBC	COSY
Phenethyl alcohol				
α	3.80 (2H, m)	71.1		
β	2.77 (2H, m)	35.4	115.9/ 130.1/ 120.0	
1	-	130.1	-	
2	6.67 (1H, *d, J* = 7.68 Hz)	117.2	-	
3	-	145.5	-	
4	-	143.3	-	
5	6.67 (1H, *d, J* = 7.68 Hz)	115.9	35.4/ 120.0/ 130.1 / 143.3/ 145.5.	
6	6.55 (1H, d, *J* = 7.81 Hz)	120.0	35.4/ 115.9/ 143.3	
Glu /1′	4.35 (1H, d, *J* = 7 Hz)	103.0	80.5/73.23	3.38
2′	3.38 (1H, m)	73.23	103.01/80.5	
3′	3.80 (1H, m)	80.5	69.1	3.38
4′	4.98 (1H, m)	69.1	66.4/ 73.23/ 166.7-80.5	3.80/3.65
5′	3.65 (1H, m)	74.13	-	3.45
6′	3.45 (1H, *dd, J* = 9.6 Hz)	66.4	-	
Rha at $C_{glu\text{-}3`}$				
1″	5.17 (1H)	102	69.33/ 71.04/ 80.5.	1.07
2″	3.91 (1H, m)	69.33	102/ 71.04	5.17
3″	3.68 (1H, m)	71.04	-	3.32
4″	3.32 (1H, m)	72.53	71.0	3.68
5″	3.58 (1H, m)	69.24	-	1.07
6″	1.07 (3H, *d, J* = 6.2 Hz)	17.2	69.24/ 72.53.	3.58
Rha at $C_{rha\text{-}2``}$				
1‴	4.61(1H)	101	66.4/ 68.6/ 71.20.	
2‴	3.83 (1H, m)	68.6	72.86/71.04	4.62
3‴	3.57 (1H, m)	71.20	72.86	3.28/3.31/4.62
4‴	3.28 (1H, m)	72.86	71.20/69.24/16.8	
5‴	3.31 (1H, m)	69.24	-	
6‴	1.18 (3H, *d, J* = 6.2 Hz)	16.8	69.24/ 72.86.	4.62
Caffeoyl moiety				
C=O	-	166.7	-	
α`	6.26 (1H, *d, J*= 15.75 Hz)	114.0	126.3/ 166.7.	
β`	7.59 (1H, *d, J* = 15.87 Hz)	147.5	114.0/ 122.0/ 166.7.	
1⁗	-	126.3	-	
2⁗	7.05 (1H, *d,*)	115.2	122.0/ 146.8/ 149.7/ 147.5.	
3⁗	-	146.8	-	
4⁗	-	149.7	-	
5⁗	6.77 (1H, *d, J* = 8.19 Hz)	115.3	126.3/ 146.8/ 149.7.	
6⁗	6.94 (1H, d, *J*= 8.06 Hz)	122.0	114.0/ 146.8/ 149.7.	

Table 4.6 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) spectroscopic data comparison of 6 with ^{1}H and ^{13}C NMR (500 MHz, CD_3OD) of teucrioside isolated from *Teucrium chamaedrys L.*

No	δ_H (600 MHz, CD_3OD) of 6	δ_C (600 MHz, CD_3OD) of 6	δ_H (500 MHz, CD_3OD) of teucrioside	δ_C (500 MHz, CD_3OD) of teucrioside
Phenethyl alcohol				
α	3.80 (2H, m)	71.1	4.05 (2H, m)	72.3 t
β	2.77 (2H, m)	35.4	2.80 (2H, t, *J* = 7.0)	36.6 t
1	-	130.1	-	131.5 s
2	6.67 (1H, *d, J* = 7.68 Hz)	117.2	6.70 (1H, d, *J* = 1.8) overlapped with H-5	116.3 d
3	-	145.5	-	146.2 s
4	-	143.3	-	144.7s
5	6.67 (1H, *d, J* = 7.68 Hz)	115.9	6.67 (1H, d, *J* = 8.0)	117.2 d
6	6.55 (1H, d, *J* = 7.81 Hz)	120.0	6.56 (1H, d, *J* = 8.0, 1.8)	121.3 d
Glu	4.35 (1H, d, *J* = 7 Hz)	103.0		
1′	3.38 (1H, m)	73.23	4.37 (1H, d, *J* = 7.9)	104.3 d
2′	3.80 (1H, m)	80.5	3.42 (1H, dd, *J* = 7.9, 9.2)	76.2 d
3′	4.98 (1H, m)	69.1	3.78 (1H, t, *J* = 9.2)	82.0 d
4′	3.65 (1H, m)	74.13	4.92 (1H, t, *J* = 9.2)	70.6 d
5′	3.45 (1H, *dd, J* = 9.6 Hz)	66.4	3.53 (1H, m)	76.1 d
6′			3.63 (2H, dd, *J* = 11.5, 2.5)	62.4 d
Rha at $C_{glu\text{-}3`}$	5.17 (1H)	102		
1″	3.91 (1H, m)	69.33	5.40 (1H, d, *J* = 1.8)	101.9 d
2″	3.68 (1H, m)	71.04	3.95 (1H, dd, J = 1.8, 3.1)	80.5 d
3″	3.32 (1H, m)	72.53	3.67 (1H, dd, J = 3.1, 9.7)	72.0 d
4″	3.58 (1H, m)	69.24	3.28 (1H, dd, J = 9.7, 9.8)	74.1 d
5″	1.07 (3H, *d, J* = 6.2 Hz)	17.2	3.53[a]	70.5 d
6″			1.07 (3H, d, *J* = 6.1)	18.6 q
Rha at $C_{rha\text{-}2``}$	4.61(1H)	101		
1‴	3.83 (1H, m)	68.6	4.88 (1H, d, *J* = 3.7)	104.3

2‴	3.57 (1H, m)	71.20	3.85 (1H, dd, *J* = 3.7, 3.6)	71.5
3‴	3.28 (1H, m)	72.86	3.71 (1H,dd, *J* = 3.6, 8.0)	72.6
4‴	3.31 (1H, m)	69.24	3.74[a]	69.0
5‴	1.18 (3H, *d, J* = 6.2 Hz)	16.8	3.68[a], 3.56[a]	65.0
6‴			-	-
Caffeoyl moiety	-	166.7		
C=O	6.26 (1H, *d, J*= 15.75 Hz)	114.0	-	168.3
α`	7.59 (1H, *d, J* = 15.87 Hz)	147.5	6.29(1H, *d, J* = 15.9)	114.7 d
β`	-	126.3	7.59 (1H, *d, J* = 15.9)	148.1 d
1‴‴	7.05 (1H, *d,*)	115.2	-	127.7
2‴‴	-	146.8	7.05 (1H, d, *J* = 2.0)	115.3
3‴‴	-	149.7	-	146.9
4‴‴	6.77 (1H, *d, J* = 8.19 Hz)	115.3	-	149.8
5‴‴	6.94 (1H, d, *J*= 8.06 Hz)	122.0	6.79 (1H, d, *J* = 8.2)	116.6
6‴‴			6.97 (1H, dd, *J* = 8.2, 2.0)	123.3

[a] (The signal pattern was unclear due to overlapping).

The ^{1}H & ^{13}C NMR data comparison of **6** with the phenylethanoid glycoside isolated from *Teucrium chamaedrys* L named teucrioside Figure 4.18 Bedir *et al* (2003)

Table 4.6 indicate that both are almost the same except the following δ values, in **6** the δ 4.62 (1H, d, *J* = 3.7, H-1```), 3.73 (1H, m, H-3```), 3.38 (1H, m, H-4```) and 3.31 (1H, m, H-5```) are shifted up field because of shielding effect resulting from the presence of an extra methyl group (CH_3-) which belongs to the second rhamnose unit, whereas in teucrioside Figure 4.19 these values are as follows 4.88 (1H, d, *J* = 3.7, H-1```), 3.71 (1H, dd, *J* = 3.6, H-3```), 3.74 (signal pattern was unclear due to overlapping) and 3.68 (signal pattern was unclear due to overlapping). Furthermore, in **6** there is an extra methyl group (CH_3-) at δ 1.18 (3H, d, *J* = 6.2, H-6```) for the second rhamnose unit which does not occur in the phenylethanoid glycoside isolated from *Teucrium chamaedrys* L named teucrioside Figure 4.18 (Bedir *et al*., 2003).

From the best of my knowledge compound **6** is a novel glycoside for which the name davaeanuside A is proposed [2-(3,4-dihydroxyphenethyl)-*O*-α-L-

rhamnopyranosyl-(1→6)-α-L-rhamnopyranosyl-(1→3)-4-*O*-*trans*-caffeoyl-β-D-glucopyranoside]. The chemical name of **6** was similar to that of teucrioside isolated from *Teucrium chamaedrys* L because most of signals in **6** are identical to those in teucrioside. It would be worthy if X-ray crystallography analysis of **6** carried out in order to have 3D structure of **6**.

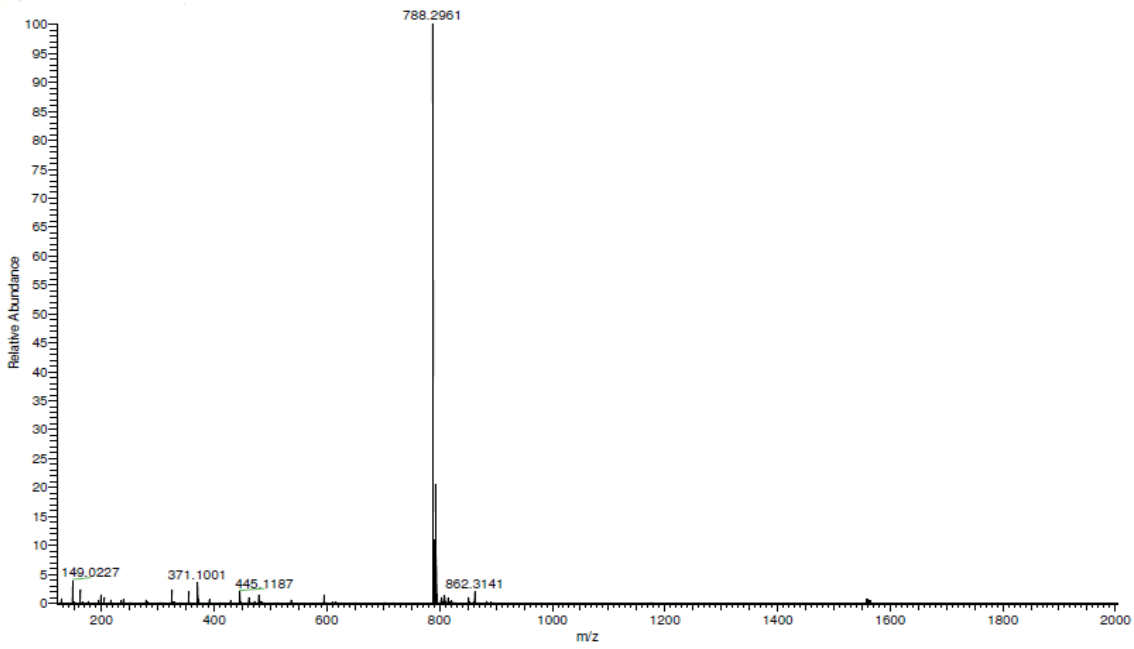


Figure 4.4 Accurate mass spectrum of 6

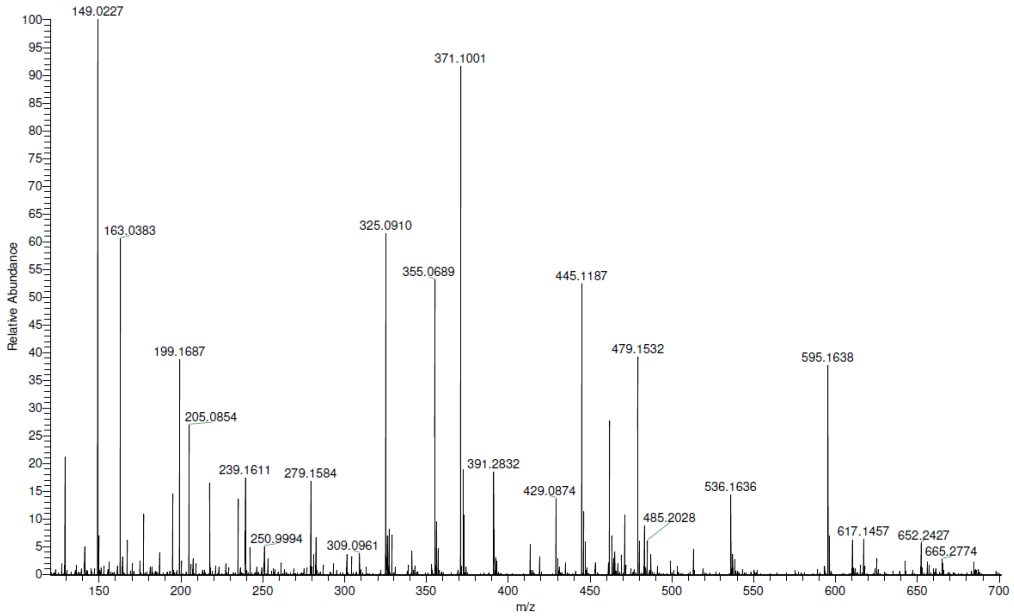


Figure 4.4 Continued.

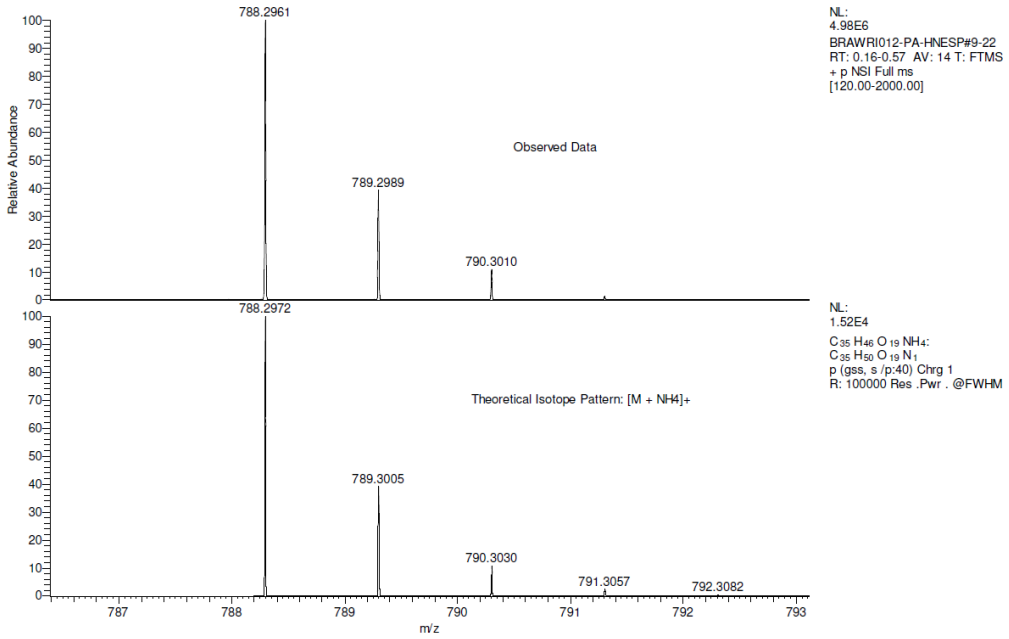


Figure 4.4 Continued

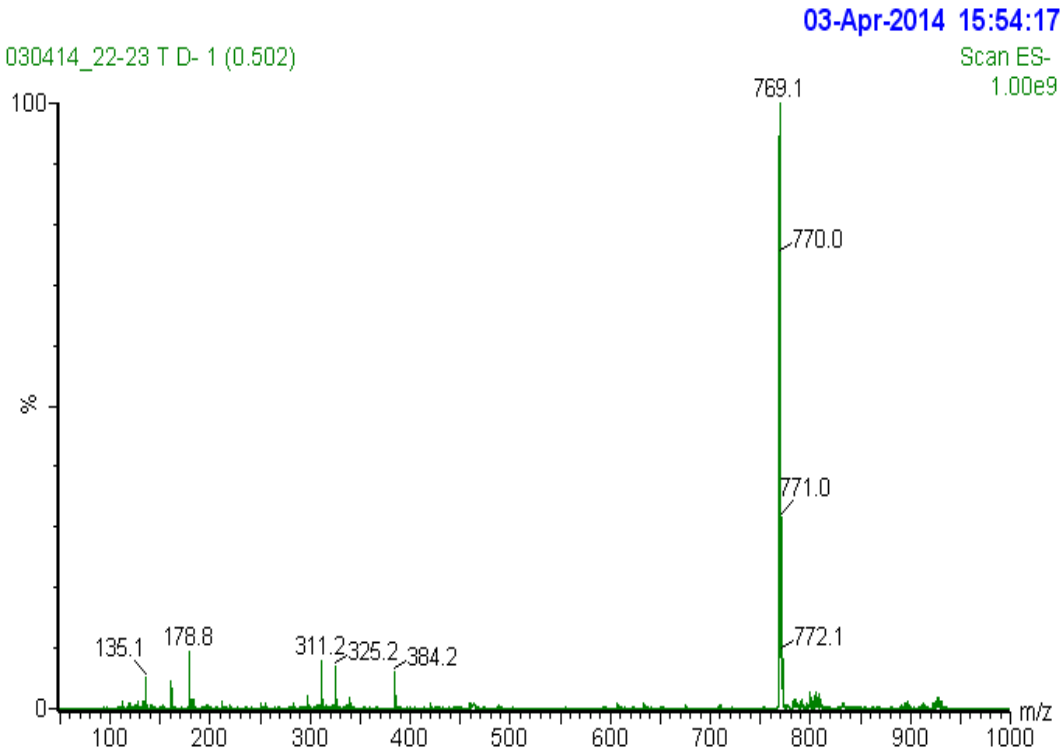


Figure 4.5 ES- mass spectrum of 6.

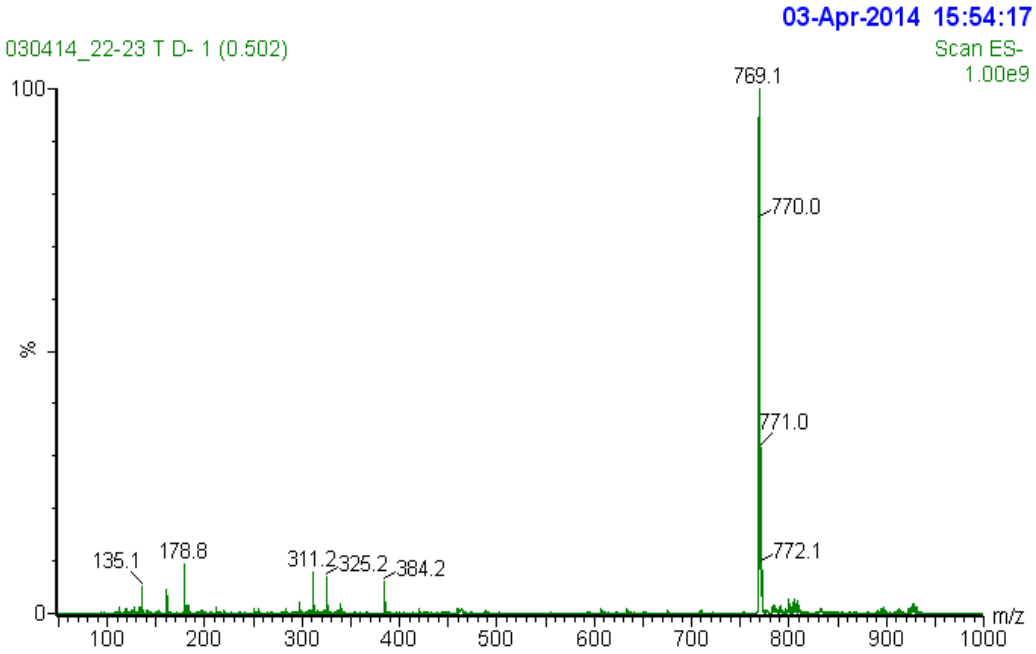


Figure 4.6 ES+ mass spectrum of 6.

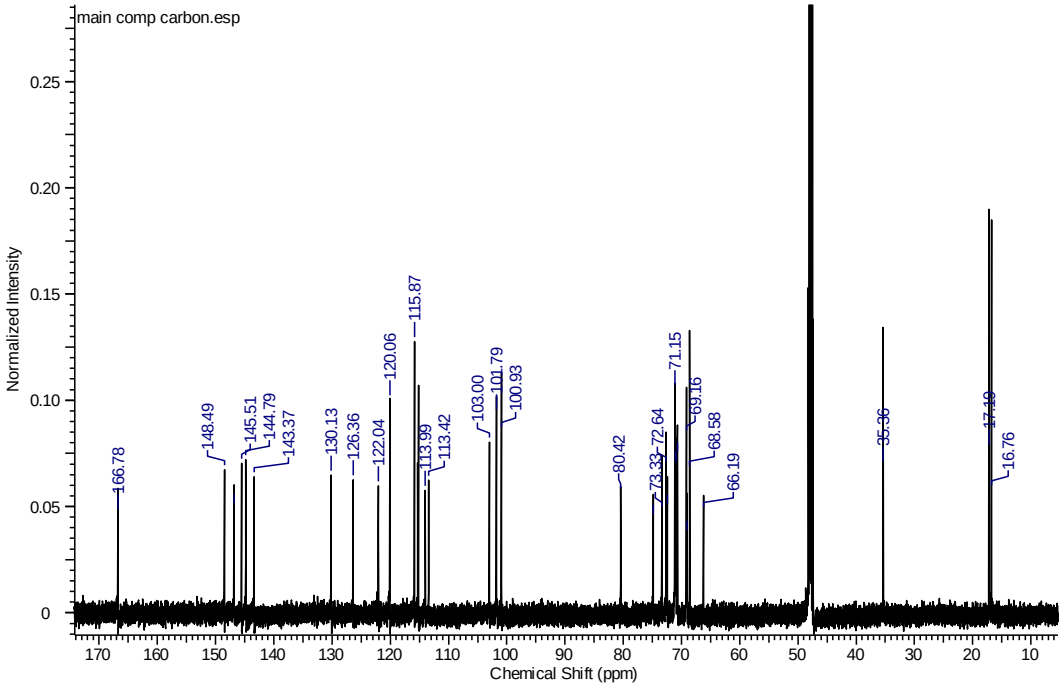


Figure 4.7 ^{13}CNMR spectrum of 6 (600 MHz, CD_3OD).

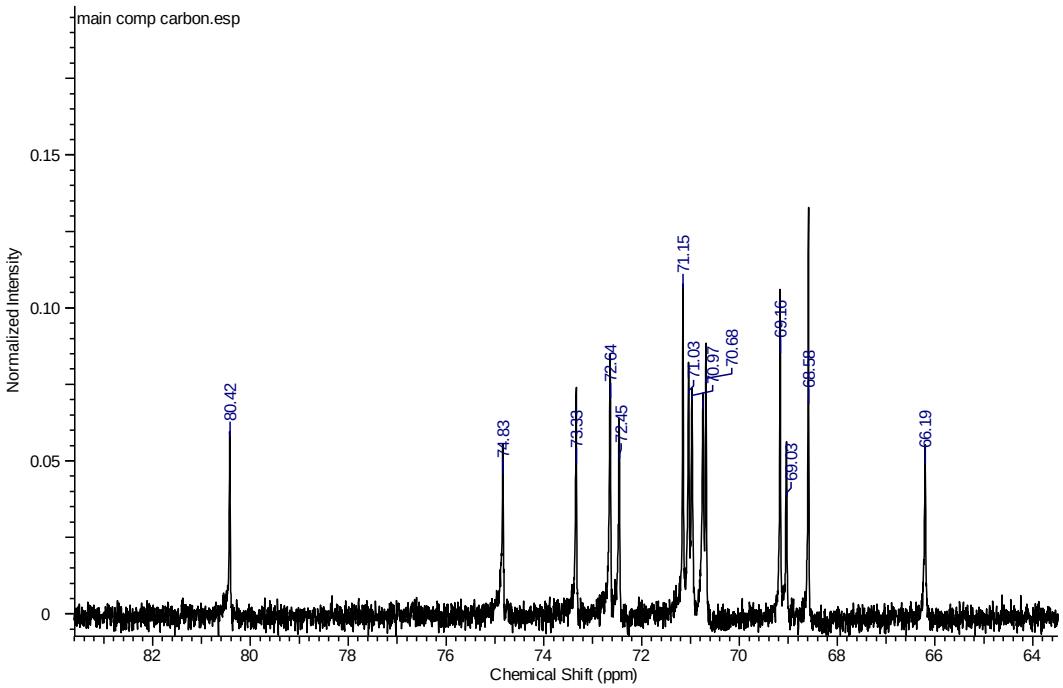


Figure 4.7 Continued.

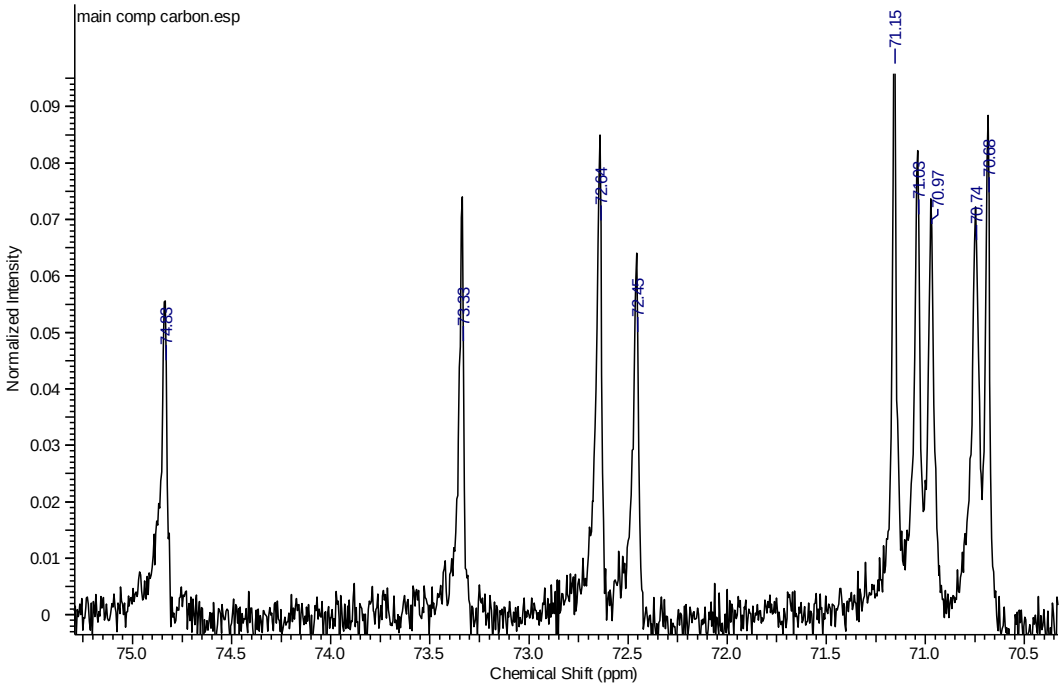


Figure 4.7 Continued.

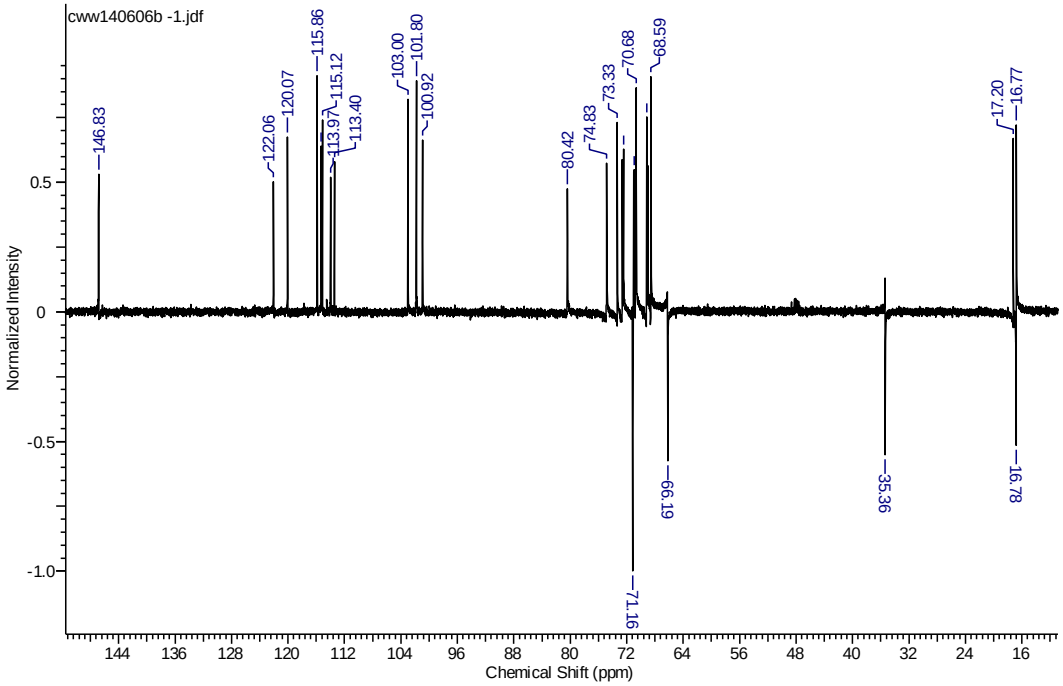


Figure 4.8 DEPT spectrum of 6 (600 MHz, CD_3OD).

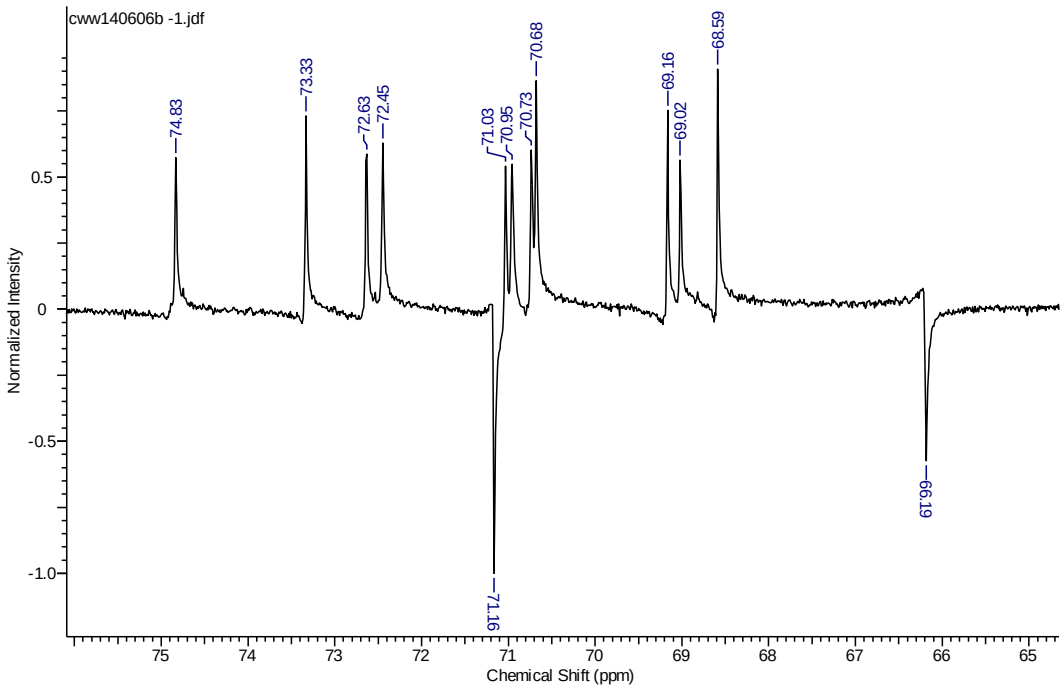


Figure 4.9 Expansion of DEPT spectrum of 6.

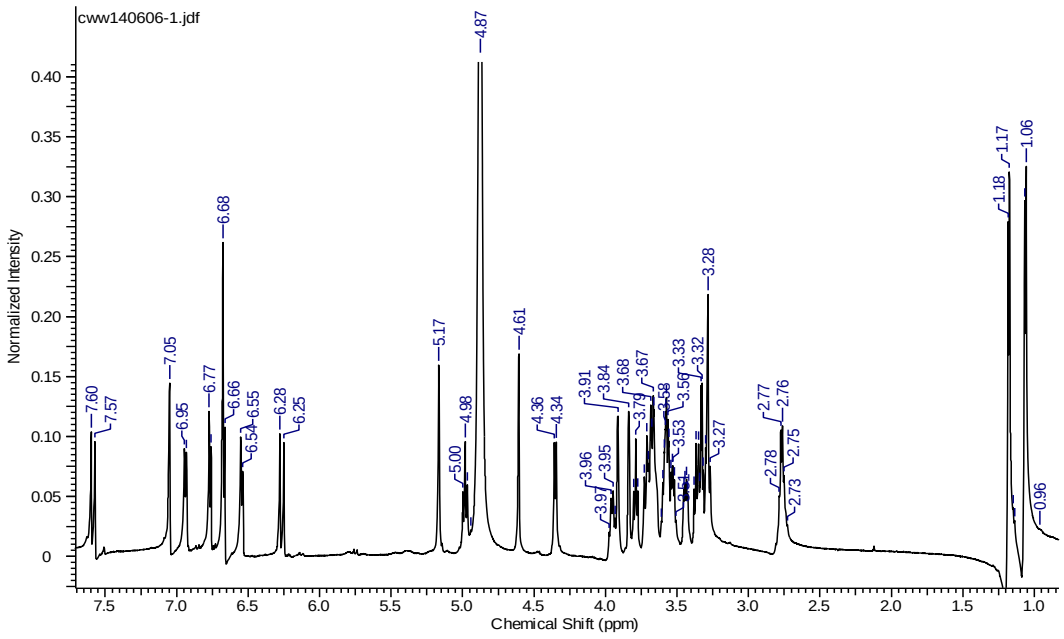


Figure 4.10 ^{1}HNMR spectrum (600 MHz, CD_3OD) of 6.

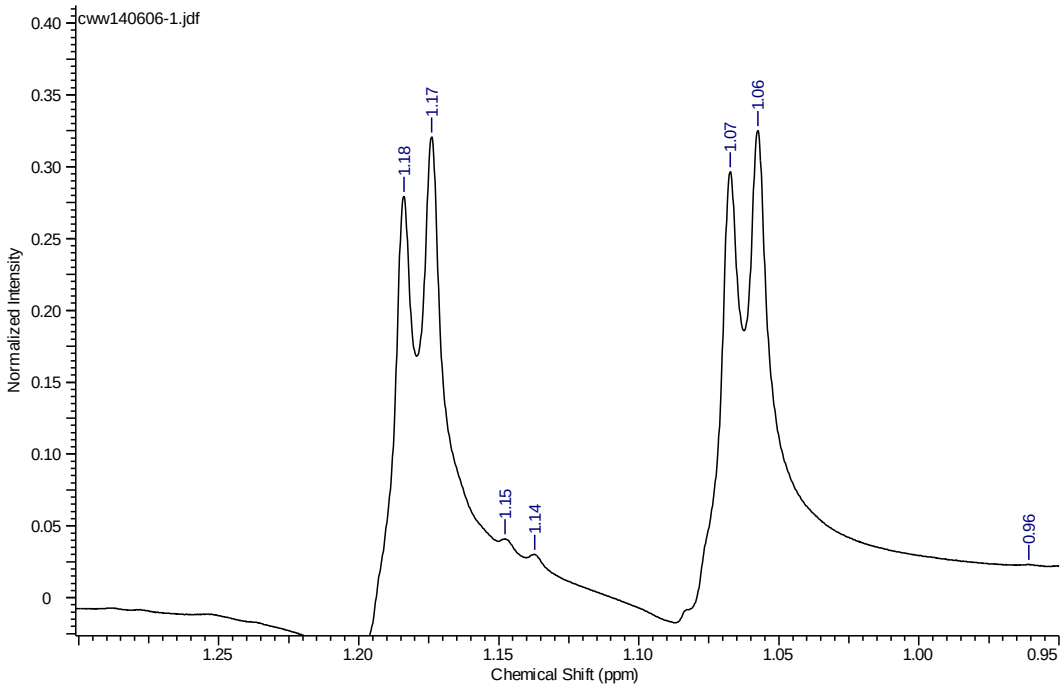


Figure 4.11 Expansion of ^{1}HNMR spectrum (600 MHz, CD_3OD) of 6.

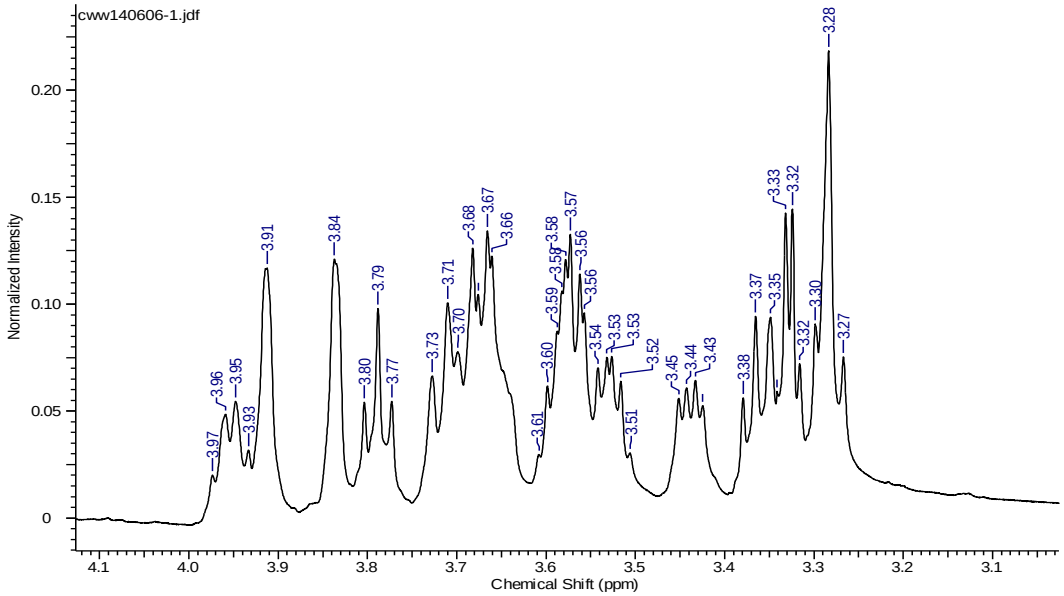


Figure 4.11 Continued.

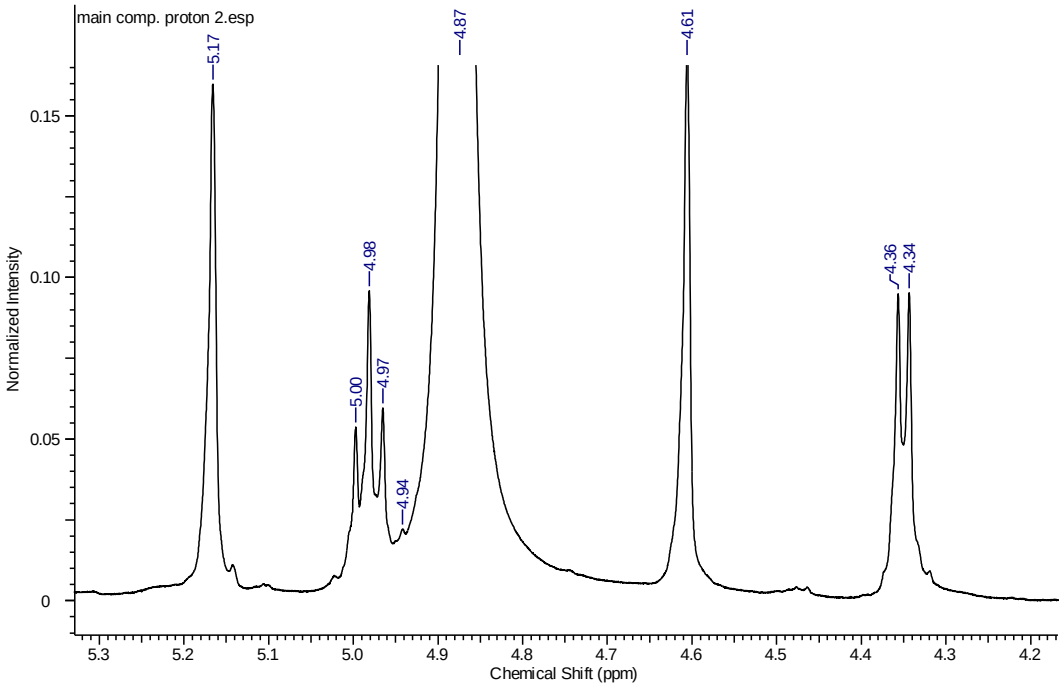


Figure 4.11 Continued.

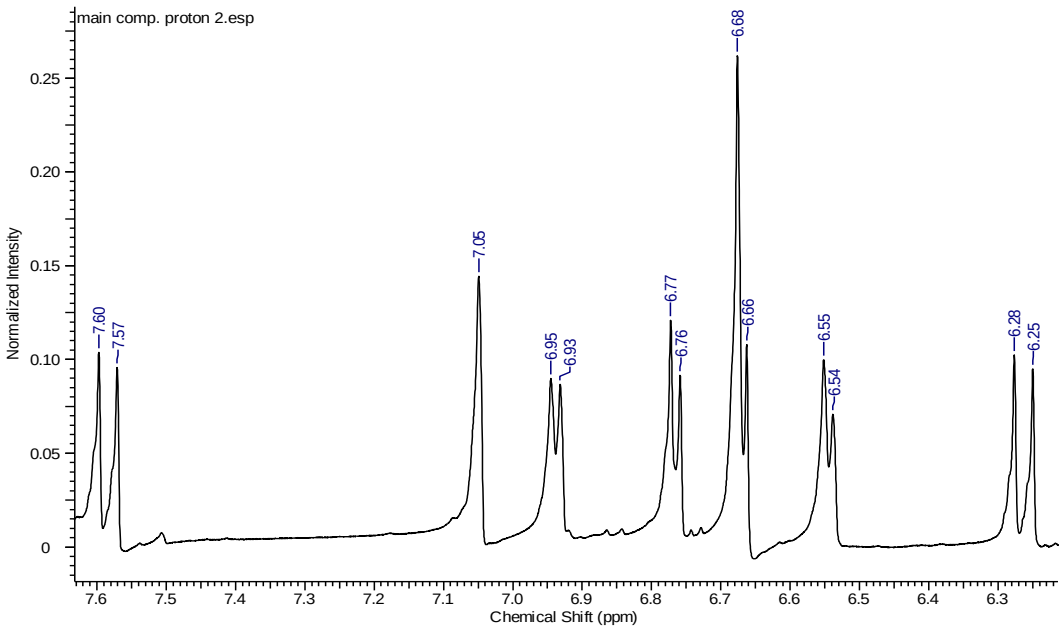


Figure 4.11 Continued.

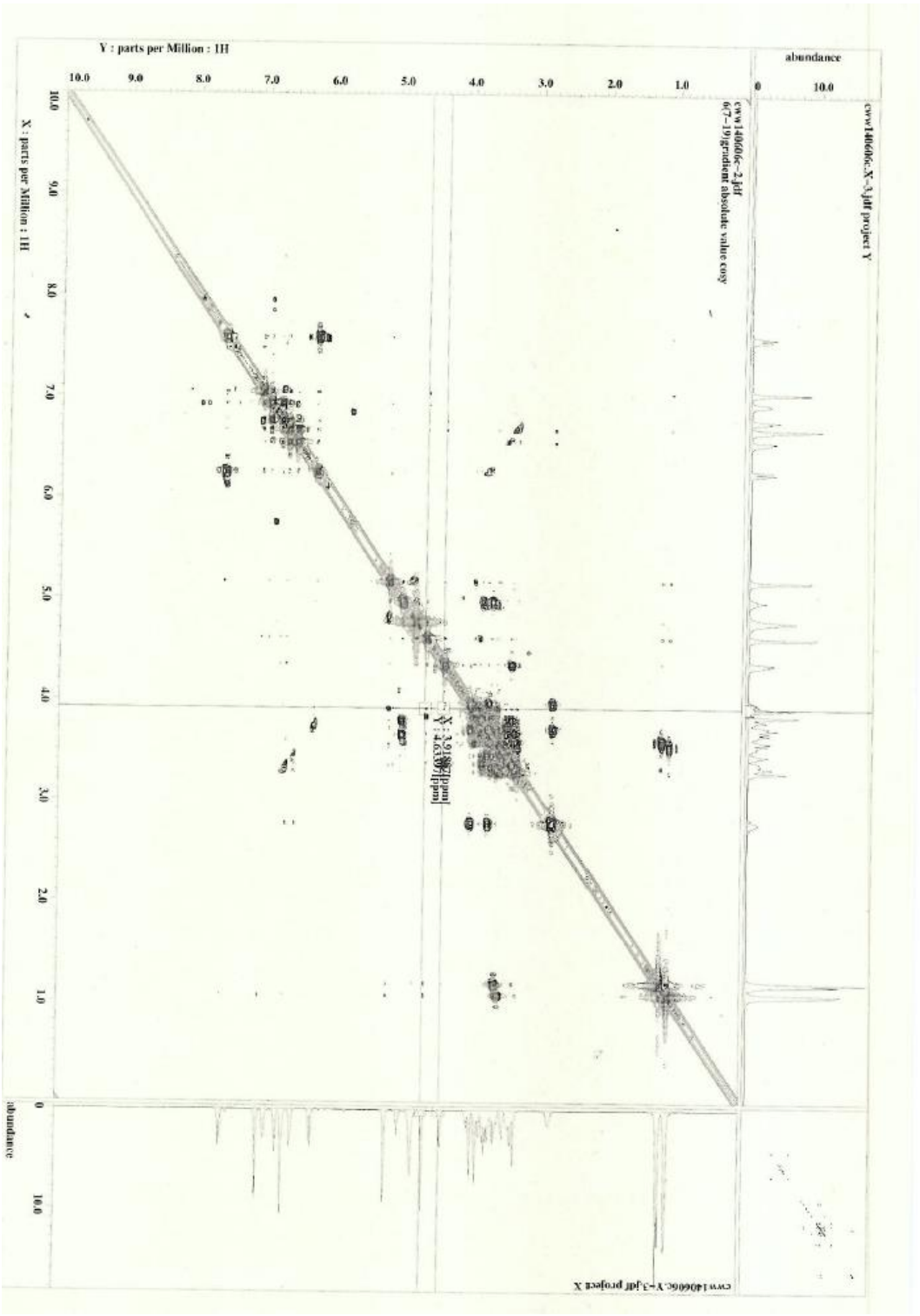


Figure 4.12 COSY spectrum (600 MHz, CD_3OD) of 6.

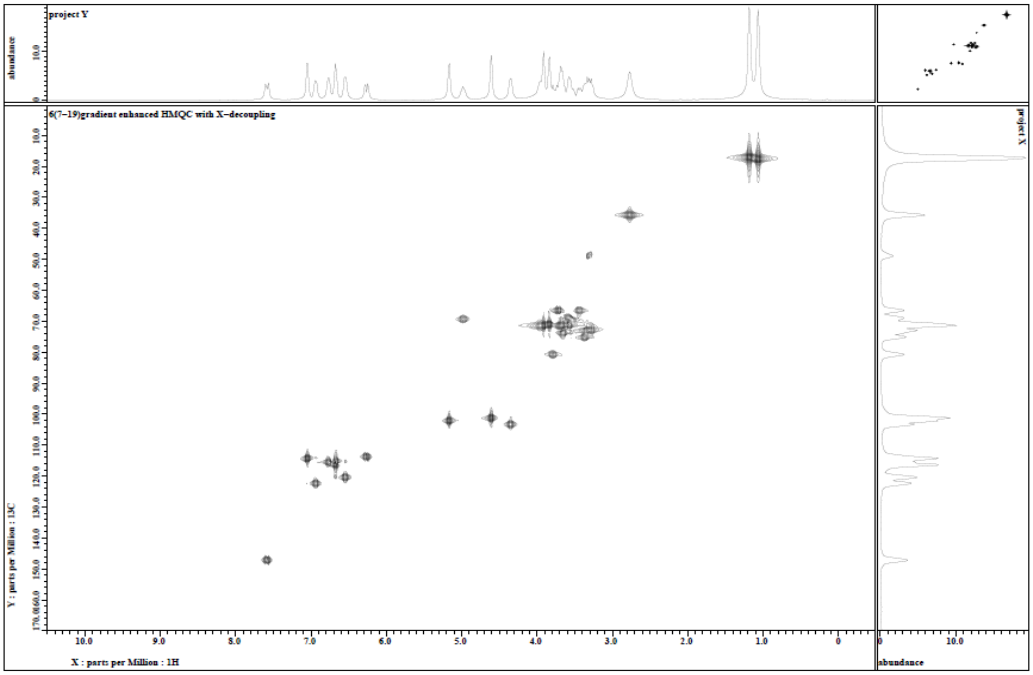


Figure 4.13 HMQC spectrum (600 MHz, CD_3OD) of 6.

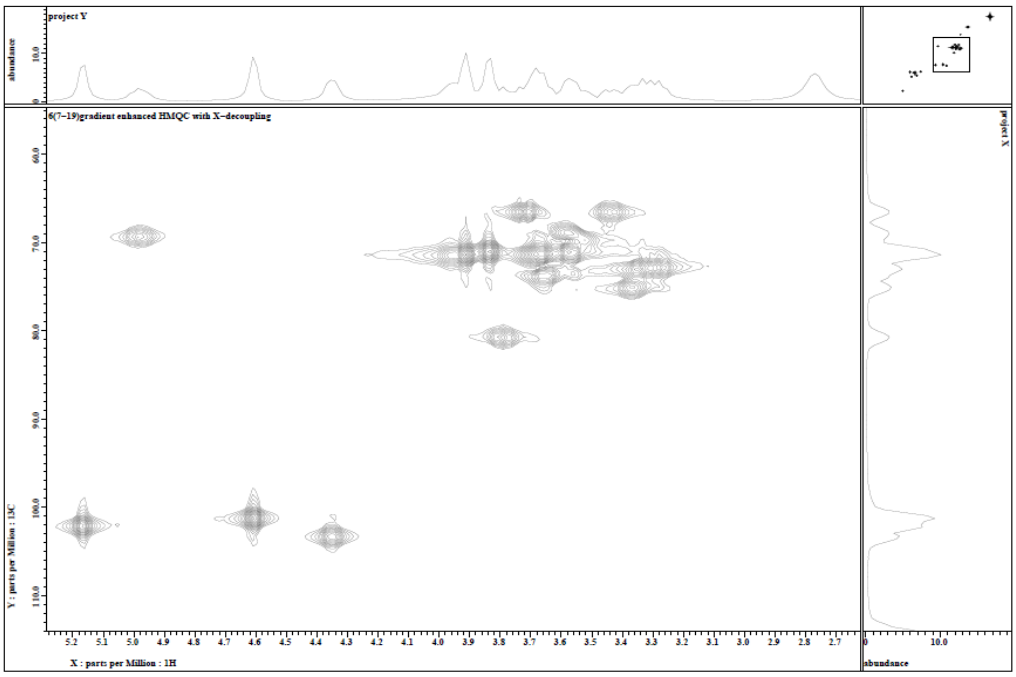


Figure 4.14 Expansion of HMQC spectrum (600 MHz, CD_3OD) of 6.

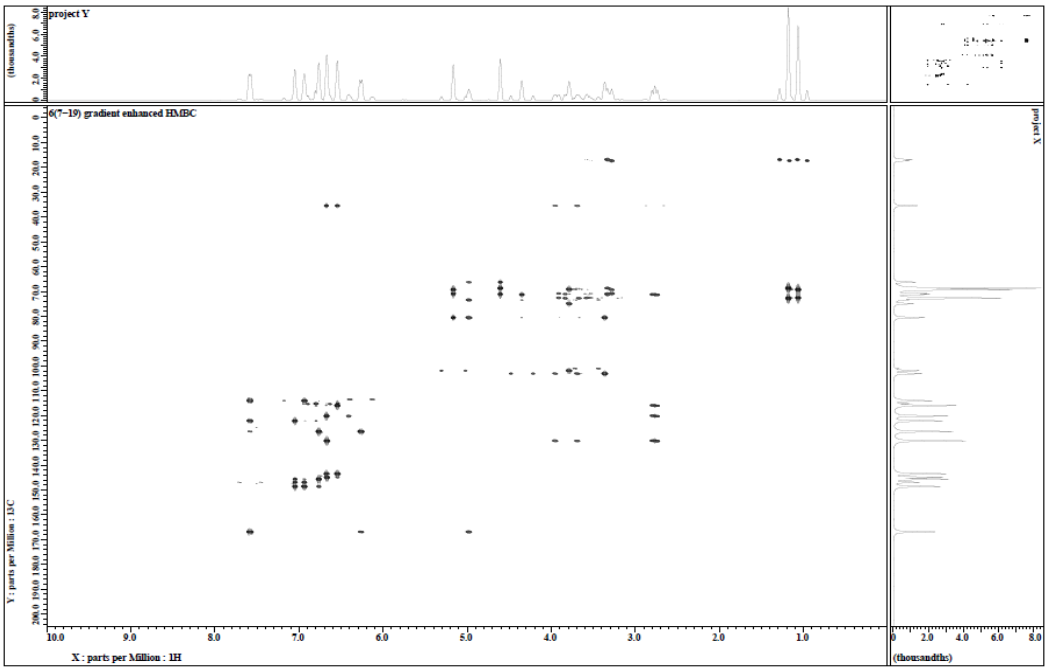


Figure 4.15 HMBC spectrum (600 MHz, CD_3OD) of 6.

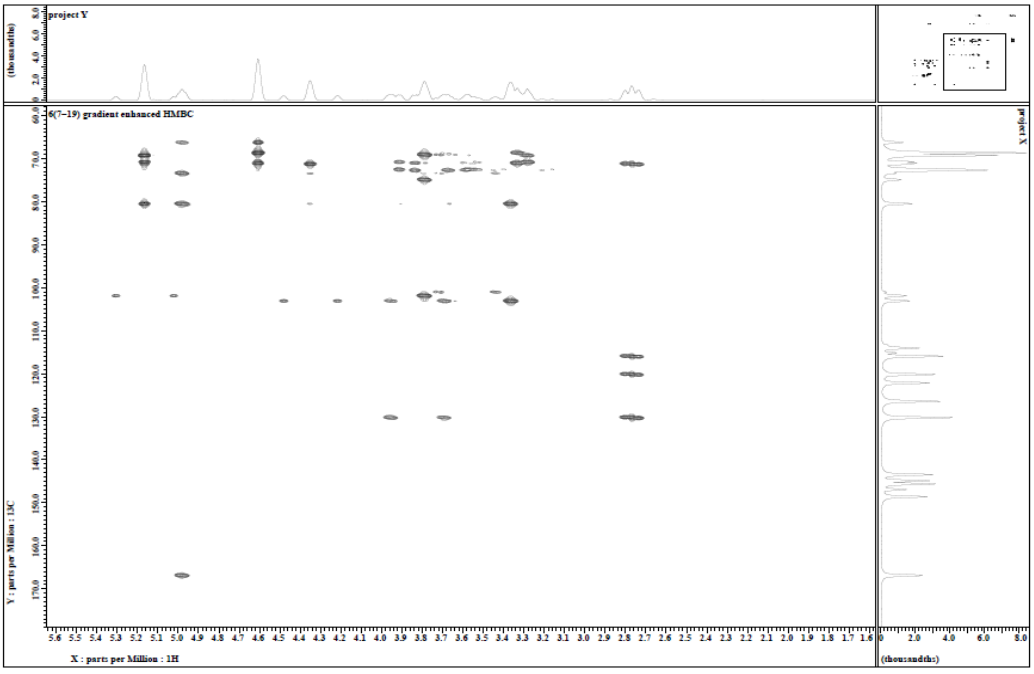


Figure 4.16 Expansion of HMBC spectrum (600 MHz, CD_3OD) of 6.

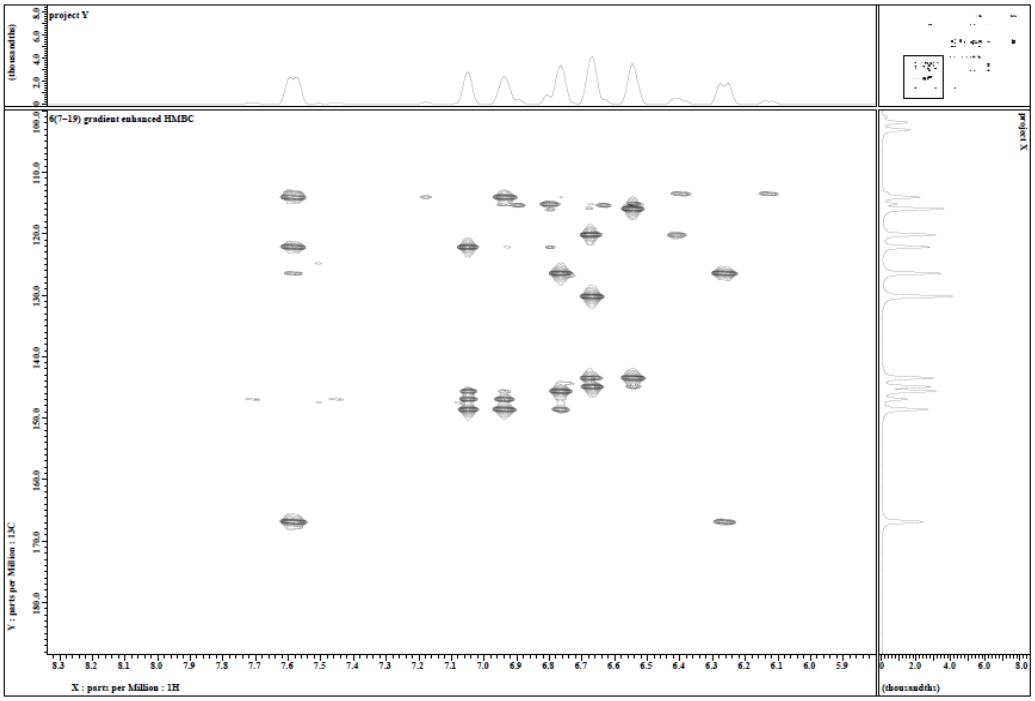


Figure 4.16 Continued.

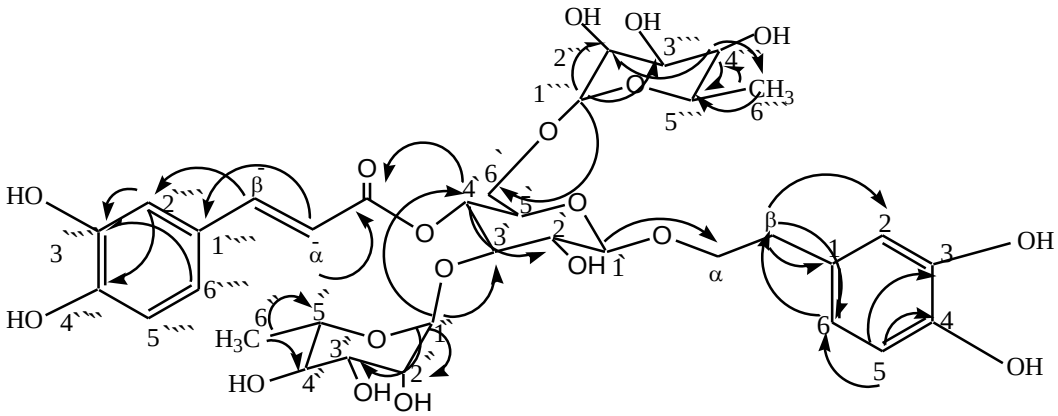


Figure 4.17 HMBC correlations of 6.

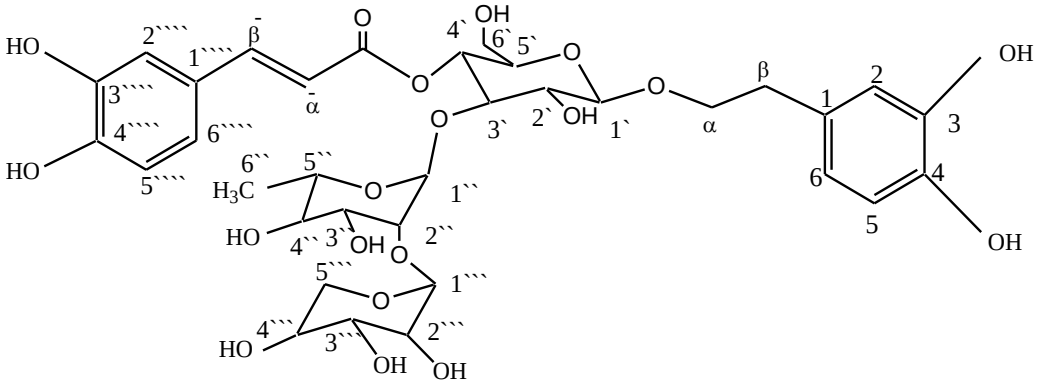


Figure 4.18 Structure of teucrioside isolated from *Teucrium chamaedrys L* (Bedir *et al*., 2003).

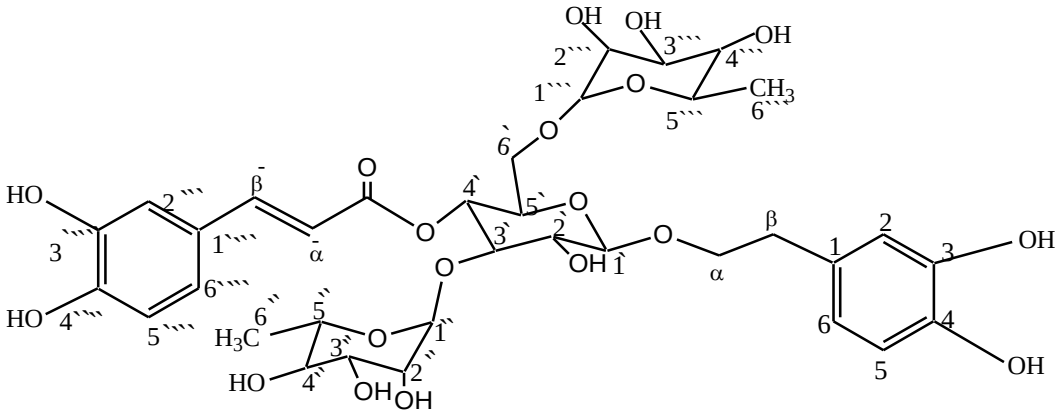


Figure 4.19 Proposed Structure of 6 isolated from *T. davaeanum L.*

4.2.3 Isolation of compounds 7 and 8

Fractions 26 and 27 Table 4.4 120 mg showed two coloured bands (green + pink) on TLC plate after spraying Figure 4.75 therefore, the fractions were mixed together and fractionated on a silica gel column as illustrated in Table 4.7.

Table 4.7 Fractionation of fractions 26-27 which contains 7 & 8.

Mobile phase	composition	Volume (ml)	Fr. Number	Combined fractions
$CHCl_3$:MeOH	100:0	200	2-3	1-20, 21
$CHCl_3$:MeOH	95:5	200	4-13	
$CHCl_3$:MeOH	90:10	200	14-23	22-26
$CHCl_3$:MeOH	80:20	200	24-33	27-29, 30-31
$CHCl_3$:MeOH	70:30	200	34-43	
$CHCl_3$:MeOH	60:40	200	44-53	42-49
$CHCl_3$:MeOH	50:50	200	54-63	50-70
$CHCl_3$:MeOH	40:60	200	64-73	
$CHCl_3$:MeOH	30:70	200	74-83	71-94
$CHCl_3$:MeOH	20:80	200	84-93	
$CHCl_3$:MeOH	10:90	200	94	
$CHCl_3$:MeOH	0:100	200	100	

Fractions [21, 22-26, 27-29, 31-41] after TLC analysis (plate conditions see section) they showed two bands with green and pink colours they were further purified using preparative TLC plates 20 plates were used to yield 77mg of compound **7** and 18 mg of compound **8**.

4.2.3.1 Partial structure elucidation of 7

Compound **7** was isolated as a sticky substance from the crude glycosides extract of *T. davaeanum*. The TLC analysis of **7** (plate conditions see

section 2.5.5) showed no UV activity at 253 nm and a green spot (Rf = 0.56) after spraying with vanillin sulphuric acid reagent (2.8.2).

The accurate mass spectrum of **7** is shown in 4.22 , which suggested a molecule of formula $C_7H_{14}O_6$ and molecular weight 184 due to the observation of $[+NH_4]^+$ and $[+Na]^+$ adducts at m/z 212 and 217 respectively. The molecular ion peak at m/z 411 is corresponding to [2M+Na] which also present in ES+ mass spectrum of **7** Figure 4.24. ES- spectrum of **7** Figure 4.25 the peak at m/z 229 could be $[M+Cl]^-$.

The ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data of **7** are shown in Table 4.8. The ^{13}CNMR spectrum of **7** (600 MHz, CD_3OD) Figure 4.26 showed the presence of 15 signals indicating that the molecule is composed from 15 carbon atoms, Figure 4.27 is shown expansion of ^{13}C NMR spectrum of 7. The DEPT spectrum of **7** (600 MHz, CD_3OD)
Figure 4.28 showed a peak at 48 which is due to the solvent (CD_3OD).

Figure 4.29 is shown expansion of DEPT spectrum of 7. The spectrum showed the presence of 4 methylene ($-CH_2-$) groups, one belonging to the glucose at δ 61.5 C-6` and the other three are belonging to the aglycone part. The spectrum showed the presence of 5 methine (-CH-) carbons for the sugar part, in which four are in δ region between 69.75 to 70.90 and one anomeric carbon C-1` at δ value of 100.1. The DEPT spectrum of **7** also gave evidence to presence of quaternary carbon because the peak at δ 103.1 (C-8) in ^{13}CNMR spectrum of **7** is absent in the DEPT spectrum.

The ^{1}HNMR spectrum of **7** (600 MHz, CD_3OD) Figure 4.30 showed the presence of an anomeric proton at δ 4.64 (1H, d, *J* = 3.72, H-1`). The other sugar proton signals are overlapped in δ region between 3.50 to 3.83. The proton signals belonging to the aglycone part appear at δ values of 4.05 (1H, d, *J* = 8.13, H-6), 3.88 (1H, t, H-5), 3.49 (2H, t, H-3) and 3.30 (2H, s, H-10). The H-3`, H-5` signals are overlapped with the other sugar

proton signals. Figure 4.31 is shown expansion of ^{1}HNMR spectrum of **7** and HMQC spectrum is shown in Figure 4.32.

The HMBC spectrum of **7** (600 MHz, CD_3OD) Figure 4.33 showed a correlations between δ 3.74 (1H, t, H-1) and the anomeric carbon C-1` at δ 100.1 which allowed the connection between the glucose anomeric carbon and C-1 of aglycone part at δ 82.1. The H-3 proton at δ 3.49 (2H, t, H-3) is correlated to C-4 at δ 63.06 which allowed the connection between C-4 at δ 63.06 and C-3 at δ 63.44. The H-4 at δ 3.58 (2H, d, H-4) is correlated to C-5 at δ 76 and to another carbon C-3 at δ 63.44 this permitted the position of C-4. The resonance of H-7 at δ 3.64 (1H, d, *J* = 3.32, H-7) is correlated to two carbon atoms at δ 103.1 C-8 and δ 76 C-5 which confirm the position of C-7 between C-8 and C-5.

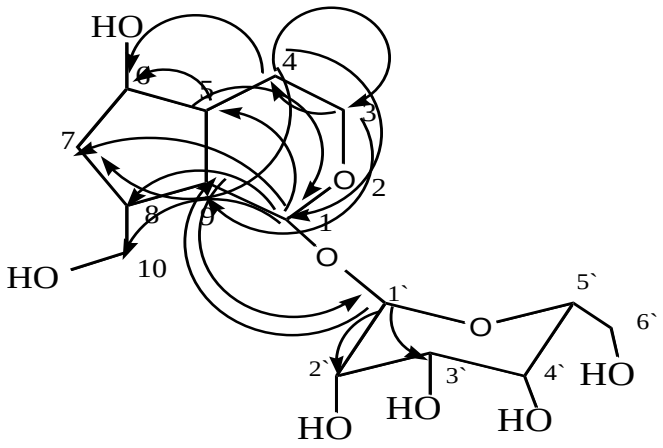


Figure 4.20 Suggested HMBC correlations of 7.

The accurate mass spectrum of **7** 4.22 indicated the presence of **7** carbon atoms, while the ^{13}CNMR Figure 4.26 showed the existence of 15 carbon atoms, therefore the $C_7H_{14}O_6$ could be the main fragment. Furthermore, the suger proton signals were overlapped which made the structure

elucidation of **7** more complicated. The glucose molecule is definitely a part of the molecule. The compound could be belonging to iridoid glycoside compounds. Further work needs to be carried out with regarding to collect a pure quantity in order to have a good 1D & 2D spectra.

Table 4.8 ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data (600 MHz, CD_3OD) of 7

No	δ^{1}H NMR (600 MHz, CD_3OD)	δ^{13}C NMR (600 MHz, CD_3OD)	DEPT (600 MHz, CD_3OD)	HMBC
1	3.74 (1H, t, H-1)	82.1	CH	60.48- 76-103.1-72.5
2	-	-	-O-	-
3	3.49 (2H, t, H-3)	63.44	CH_2	63.06-54.4
4	3.58 (2H, d, H-4)	63.06	CH_2	82.1-63.44-103.1-72.5-76-78
5	3.88 (1H, t, H-5)	76	CH	78-63.44-82.1
6	4.05 (1H, d, *J* = 8.13 H-6)	78	CH	76-60.21-103.1
7	3.64 (1H, d, *J* = 3.32, H-7)	72.5	CH	103-78-63.31
8	-	103.1	Q	-
9	3.44 (1H, d, H-9)	54.4	CH	100.1
10	3.30 (2H, s, H-10)	60.21	CH_2	103.1
1`	4.64 (1H, d, *J* = 3.72, H-1`)	100.1	CH	54.4-70.30-71.43
2`	3.65 (1H, t, H-2`)	71.43	CH	-
3`	3.87(the signal overlapped)	69.75	CH	69.96-70.28
4`	3.69 (1H, t, H-4`)	72.56	CH	69.96-70.93
5`	3.70 (the signal overlapped)	69.96	CH	-
6`	3.68 (1H, d, J = 2.75, H-6`)	61.46	CH_2	69.79

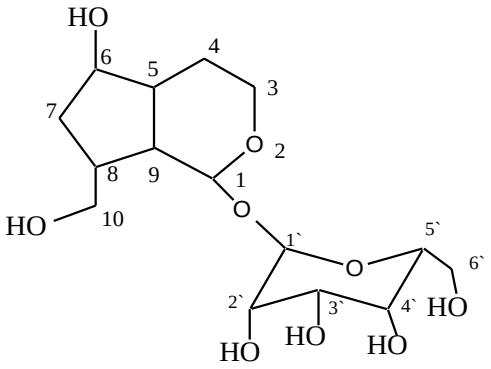


Figure 4.21 Suggested structure of 7.

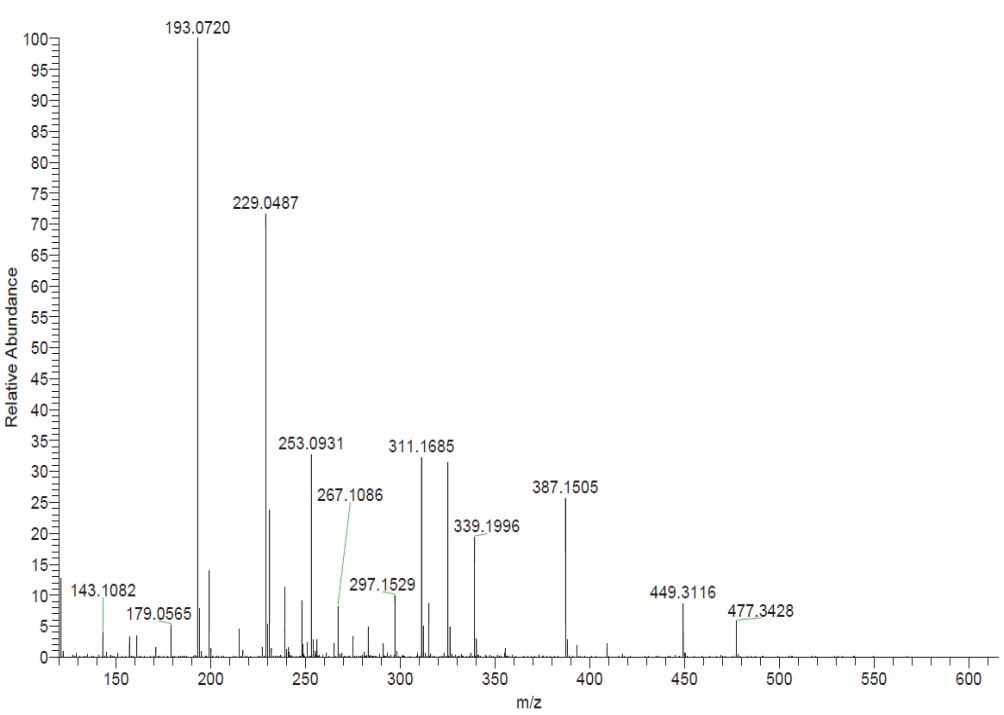


Figure 4.22 An accurate mass spectrum of 7.

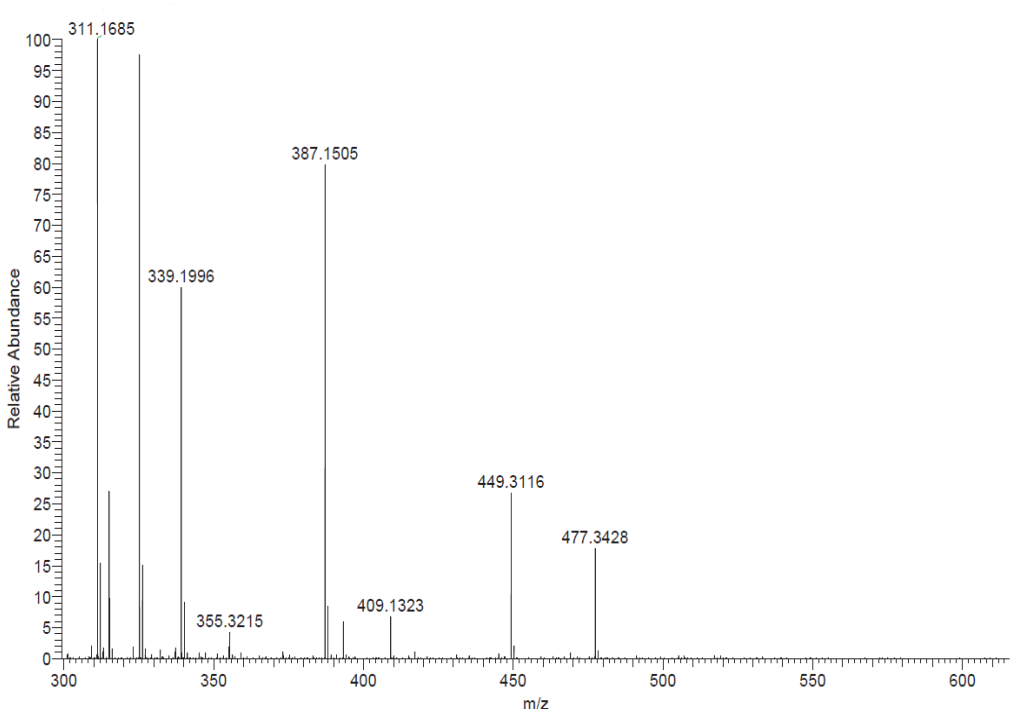


Figure 4.23 Expansion of accurate mass spectrum of 7.

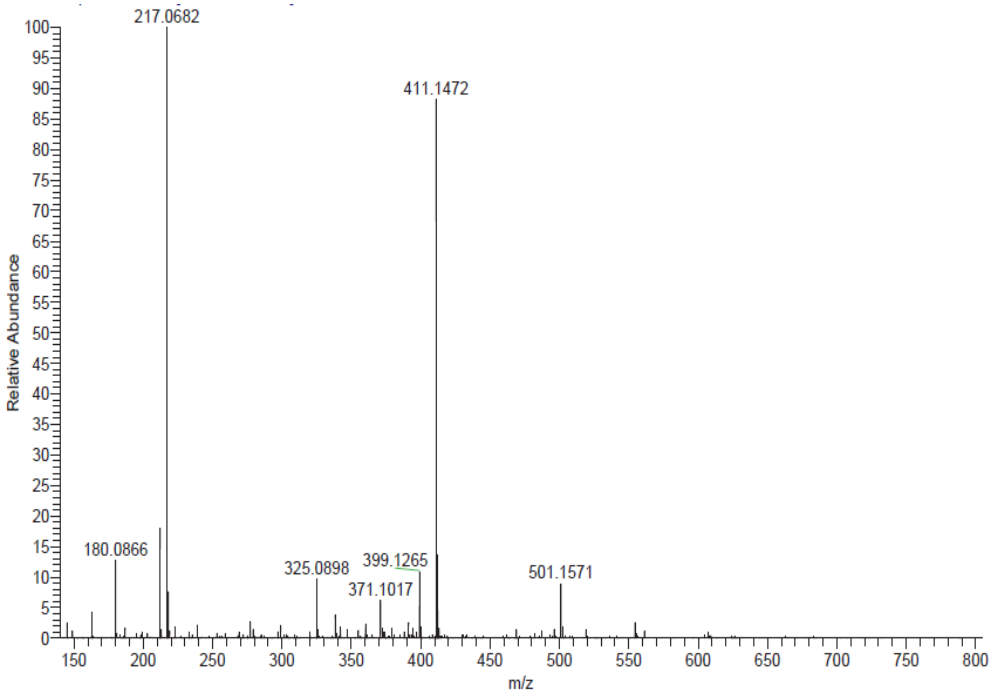


Figure 4.23 Continued.

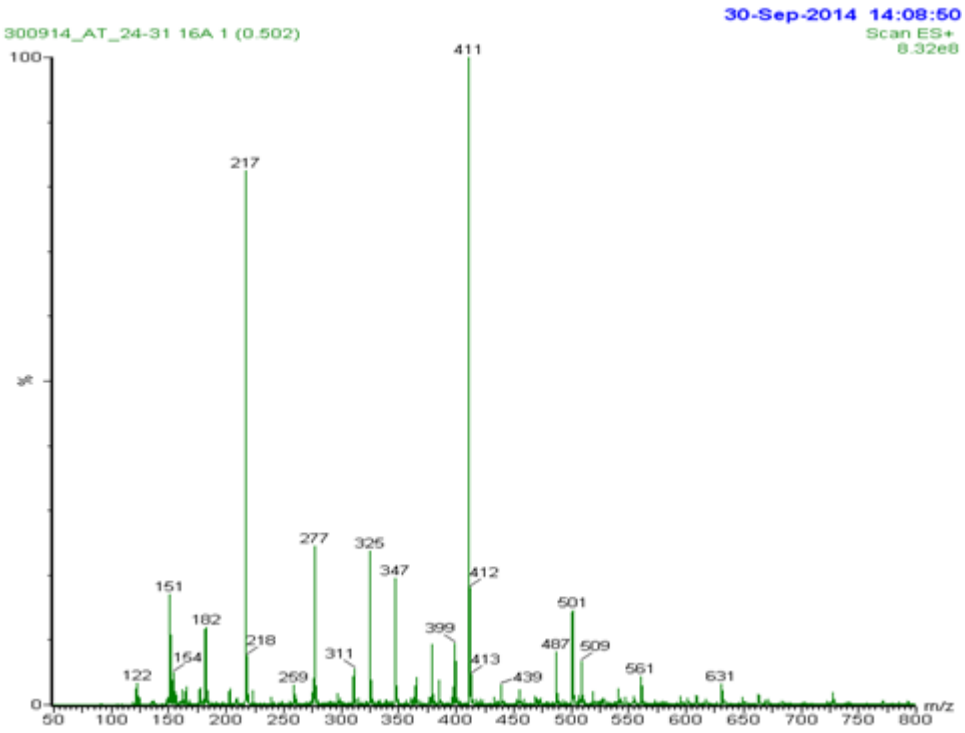


Figure 4.24 ES+ spectrum of 7.

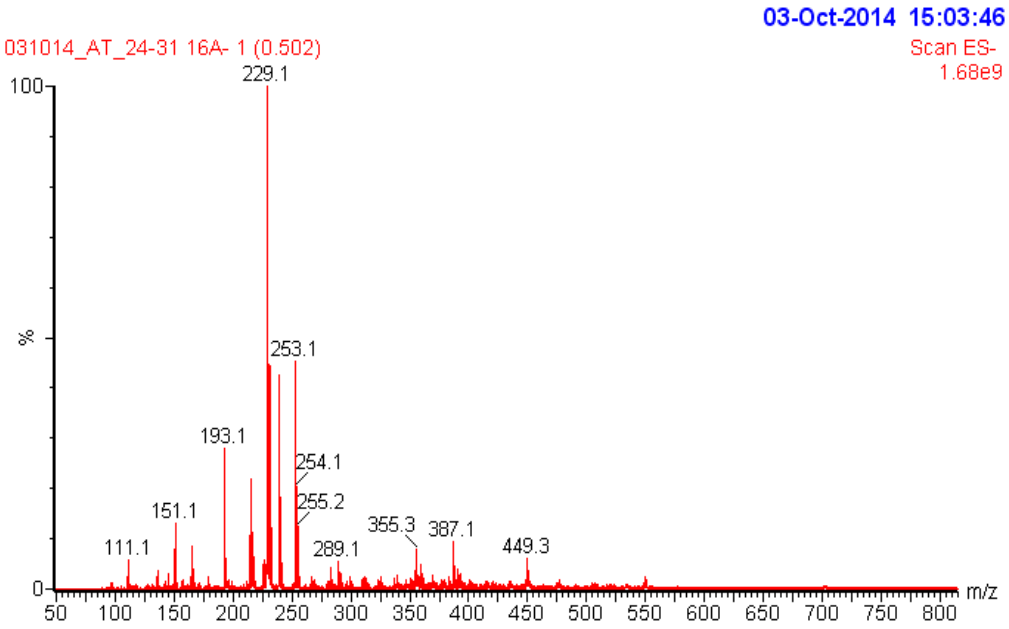


Figure 4.25 ES- mass spectrum of 7.

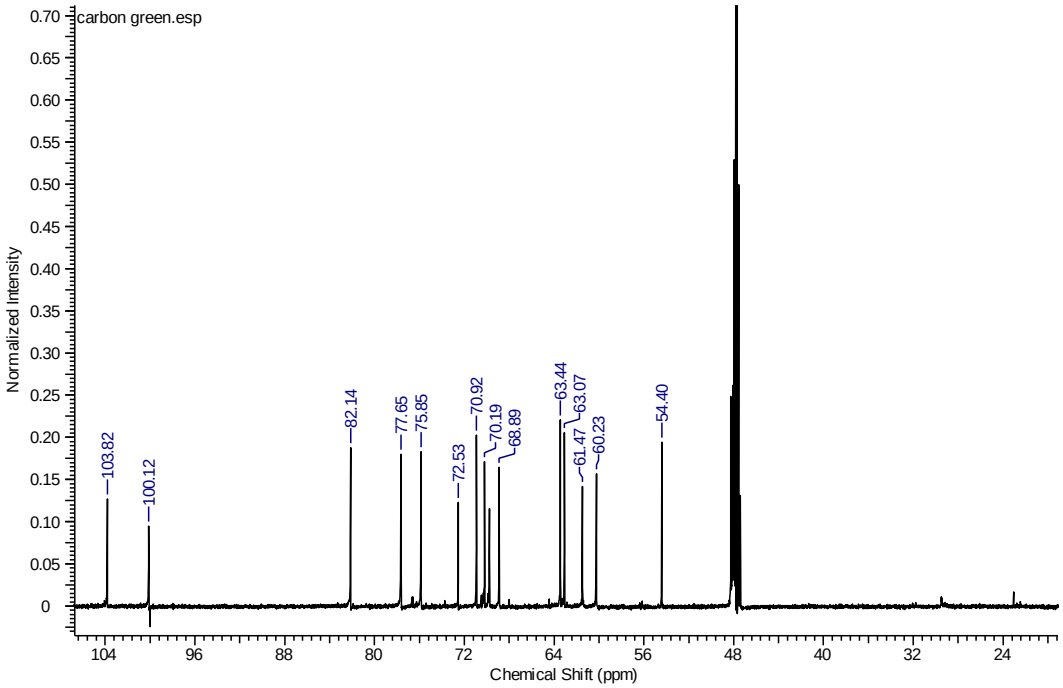


Figure 4.26 ^{13}C NMR spectrum (600 MHz, CD_3OD) of 7.

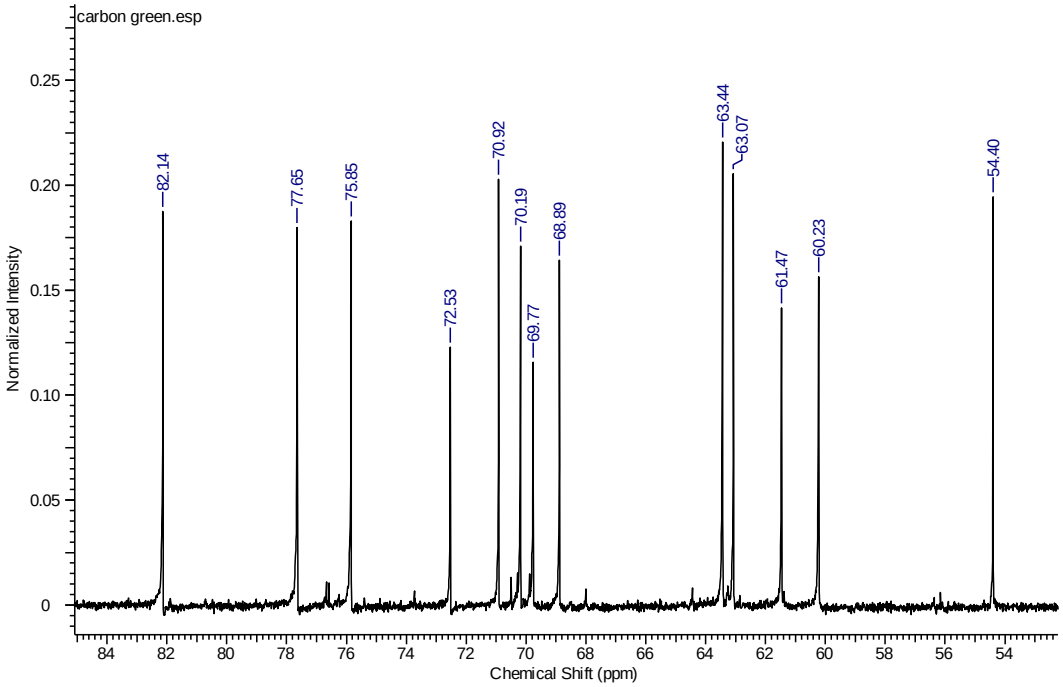


Figure 4.27 Expansion of ^{13}C NMR spectrum (600 MHz, CD_3OD) of 7.

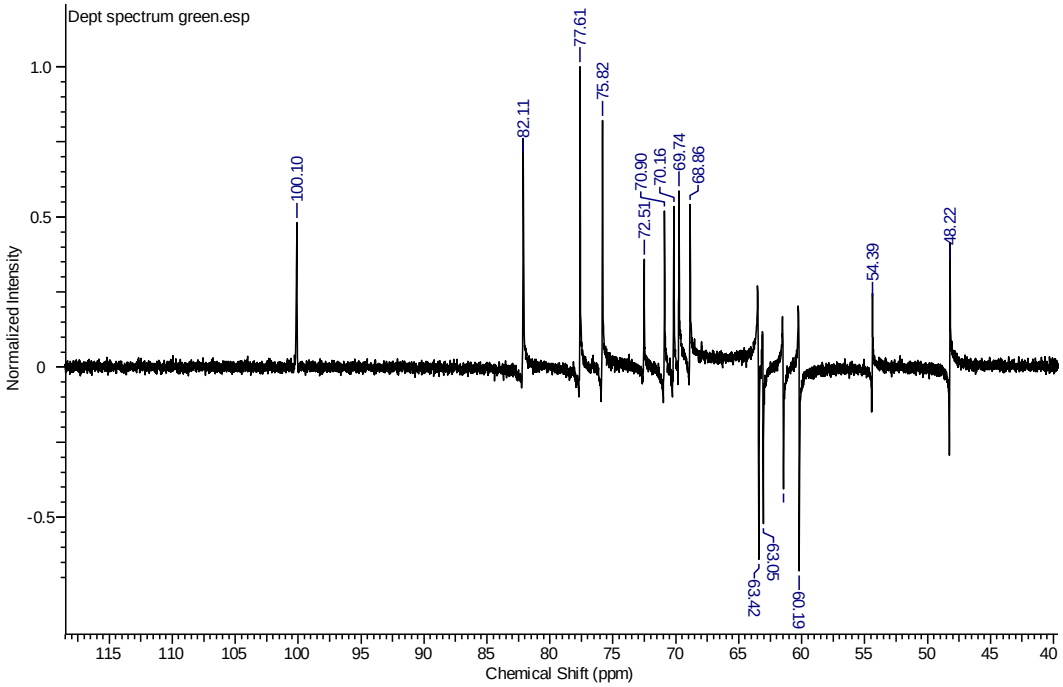


Figure 4.28 DEPT spectrum of 7.

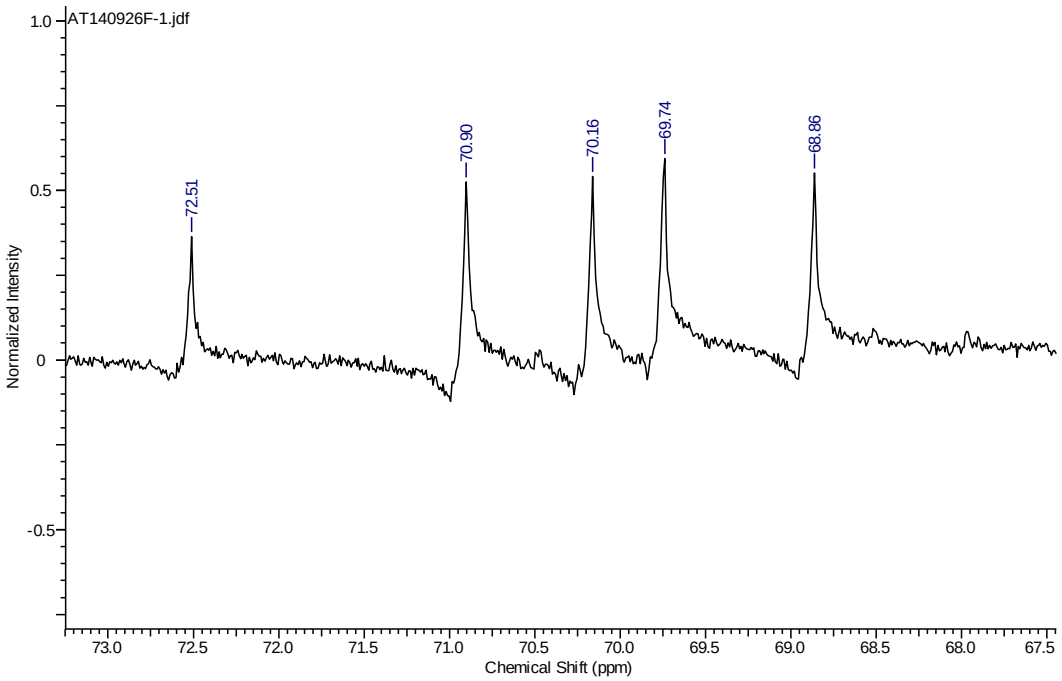


Figure 4.29 Expansion of DEPT spectrum of 7.

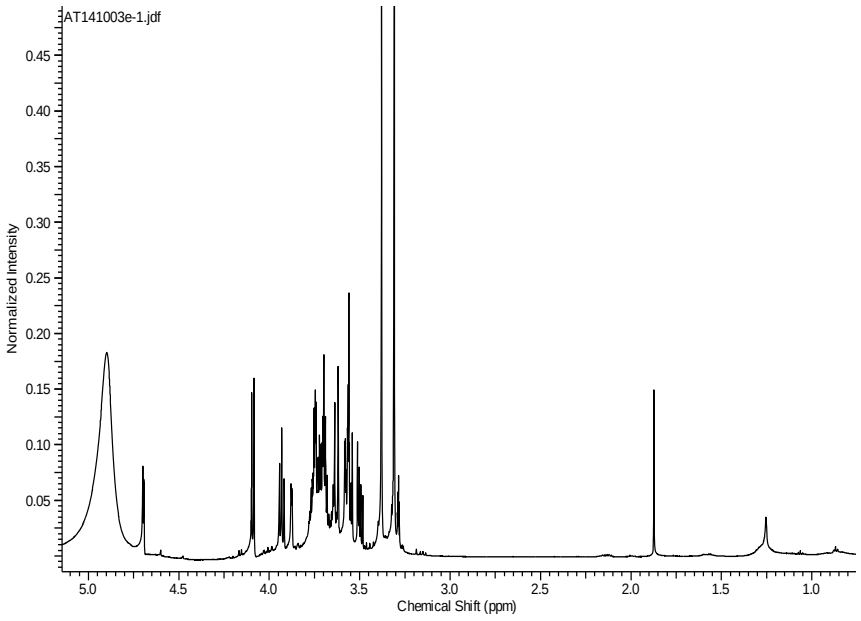


Figure 4.30 ^{1}H NMR spectrum (600 MHz, CD_3OD) of 7.

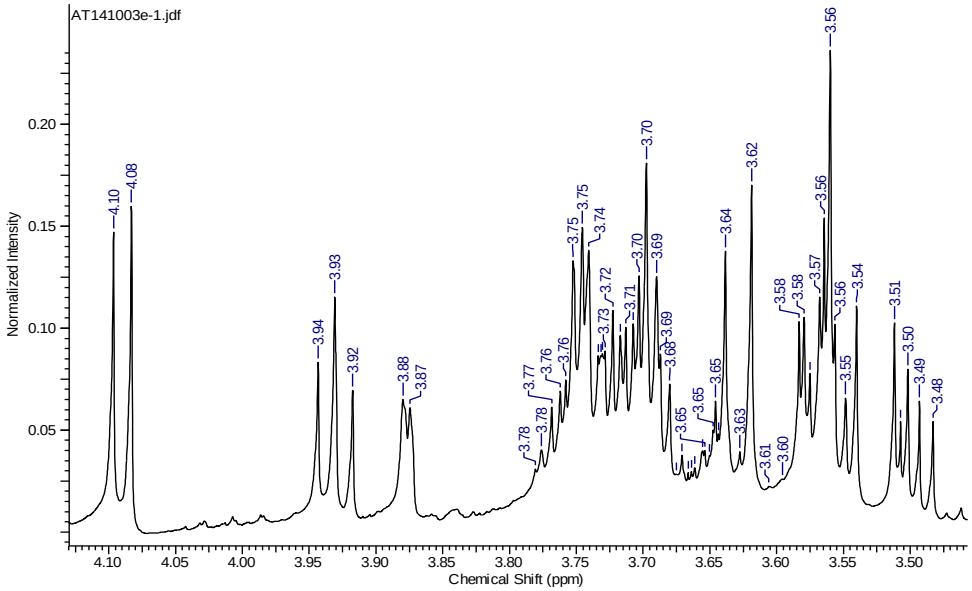


Figure 4.31 Expansion of ^{1}H NMR spectrum (600 MHz, CD_3OD) of 7.

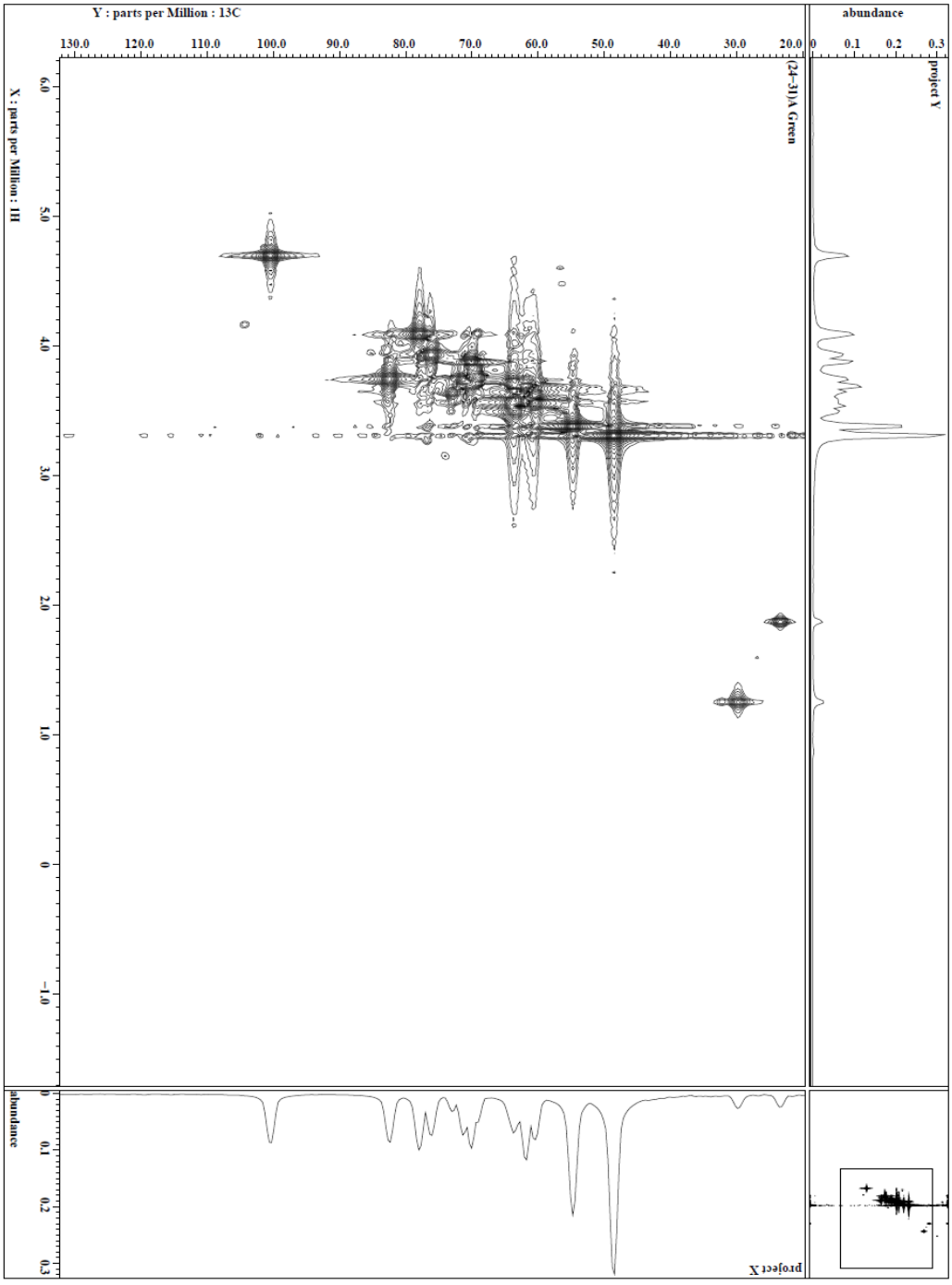


Figure 4.32 HMQC spectrum (600 MHz, CD_3OD) of 7.

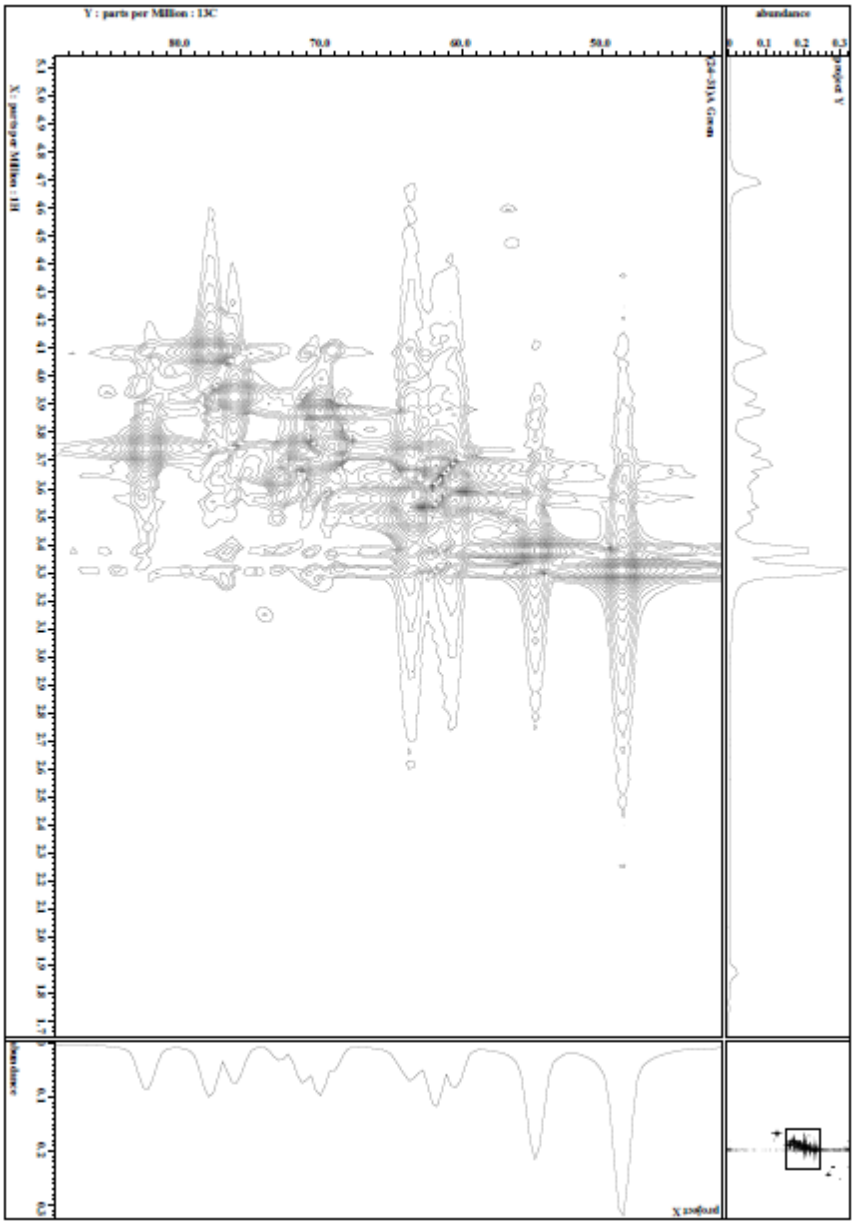


Figure 4.32 Continued.

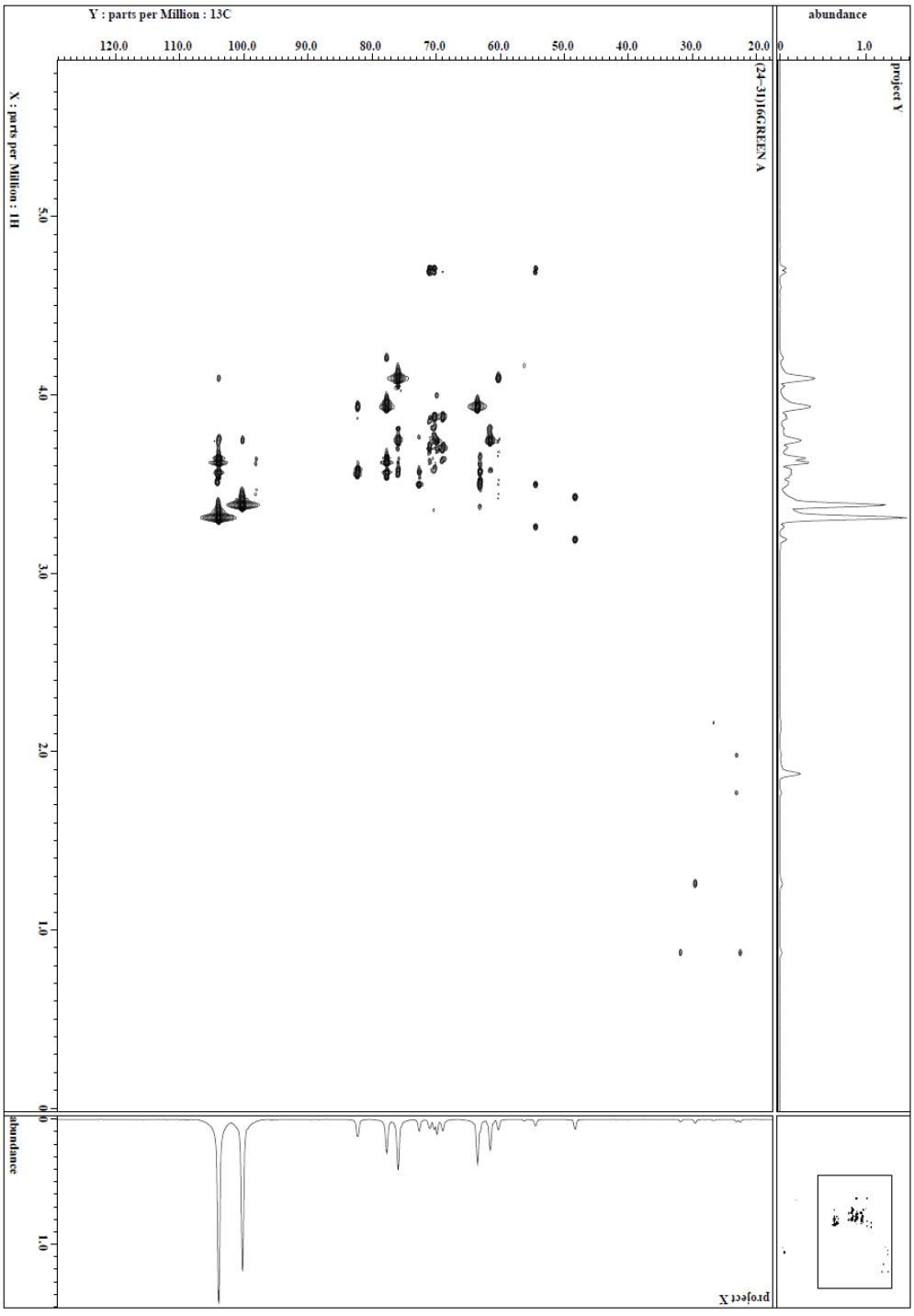


Figure 4.33 HMBC spectrum of 7.

4.2.3.2 Structure elucidation of compound 8 (pink compound)

Compound **8** was isolated as a brown sticky substance from the crude glycosides extract of *T. davaeanum*. The TLC analysis of **8** (2.5.5) showed UV activity at 253 nm and a pink spot (Rf = 0.64) after spraying with vanillin sulphuric acid reagent (section 2.8.2). Compound 8 has the molecular formula $C_{17}H_{28}O_{11}$ (m/z = 406.1) as determined by an accurate mass measurement, the measured accurate mass was 424.1813 and the calculated accurate mass was 424.1813 corresponding to the molecular ion peak $[M + NH4]^+$ Figure 4.35 . The ES+ spectrum of **8** Figure 4.36 showed a peak at m/z = 429.1 $[M + Na]^+$. The ES- spectrum of **8** Figure 4.37 displayed a peak at 441.0 $[M + Cl]^-$.

The ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data of **8** are shown in Table 4.9. The ^{13}CNMR spectrum of **8** (600 MHz, CD_3OD) Figure 4.38 showed the presence of 17 signals indicated the molecule has 17 carbon atoms. The up field region of the spectrum showed a presence of two methyl groups at δ 20.9 C-10 and δ 21.2 C-12, also there are two methylene groups of aglucone part (-CH_2-) of C-6 and C-7 at δ 29.5, 44.8 respectively. The methine group appears at δ 54.1 C-9. The sugar –CH- carbons displayed at δ values of 70.9 C-3`, 73.8 C-5`, 76.3 C-2` and 76.9 C-4`, while the –CH_2- appears at δ 61.7 C-6` and the anomeric carbon at δ 98.1 C-1`. The -HC=CH- carbons displayed at δ 142.6 C-3 and δ 105.5 C-4 and, while the carbonyl carbon was shown at δ 172.1 C-11.

The DEPT spectrum of **8** (600 MHz, CD_3OD) Figure 4.39 showed a presence of three methylene groups (-CH_2-), two are belonging to aglycone part, appears in upfield region at δ 29.5 C-6 and 44.8 C-7, and the third one is belonging to sugar part displayed in downfield region at δ 61.7 C-6`.

The ^{1}HNMR spectrum of **8** (600 MHz, CD_3OD) Figure 3.39 showed signals of two methyl groups at δ 1.37 (3H, s, H-10) and at δ 1.94 (3H, s,

H-12). Also two methylene (-CH_2-) groups at δ 1.27 (2H, t, H-6) and 2.10 (2H, t, H-7). The H-9 proton appears at δ 2.81 (1H, s, H-9), while signals for the sugar protons were found at δ 3.18 to 3.85. The down field region of the spectrum showed the presence of two (-CH-) groups one of the anomeric proton at δ 4.56 (1H, d, *J* = 7.84, H-1`) and the second is connected to the C-1 at δ 6.04 (1H, d, H-1), Figure 4.41 is shown expansion of ^{1}HNMR spectrum of **8**. The H-3 proton appears at δ 6.36 (1H, d, *J* = 6.35, H-3), H-4 proton showed at δ 4.89, the signal is hidden under the signal of hydroxyl; two evidence indicated the presence of this signal, in the COSY spectrum Figure **4.42** the proton at δ 4.89, H-4 is coupled to the proton at δ 6.36 (1H, d, *J* = 6.35, H-3) meaning that the two protons are adjacent. Therefore the signal at δ 4.89 is expected to be doublet, also in the HMQC spectrum Figure 4.43 showed a presence of protin at δ 4.89 connected to the carbon atom at δ 105.5. A distinctive signal at δ 4.82 is related to the solvent hydroxyl group, also it could be related to water hydroxyl group; due to the presence of water in solvent, the sample is not completely dried or a damp NMR tube.

The HMBC spectrum of **8** Figure 4.44 showed the following correlations, the cross peak from the anomeric proton of glucose δ 4.56 (1H, d, *J* = 7.84, H-1`) to the carbon atom C-1 at δ 93.2 of the aglycone part allowed the connection of glucose anomeric carbon to C-1 of aglycone part. The DEPT spectrum of **8** showed the presence of two quaternary carbons at δ 72 C-5 and δ 87.4 C-8. The H-1 at δ 6.04 (1H, d, H-1) has correlation with C-3 δ 142.6, C-1` δ 98.1 and C-5 δ 72, also H-3 at δ 6.36 (1H, d, *J* = 6.35, H-3) is correlated to C-5 δ 72 this allowed the positions of C-5. Furthermore, the H-12 proton at δ 1.94 (3H, s, H-12) are correlated only to the carbon atom at δ 172.1 C=O allowed the connection between C-12 δ 21.2 and carbonyl group C-11 δ 172.1, the other correlations are summarized in Table 4.9. Figure 4.34 is shown HMBC correlations of **8**.

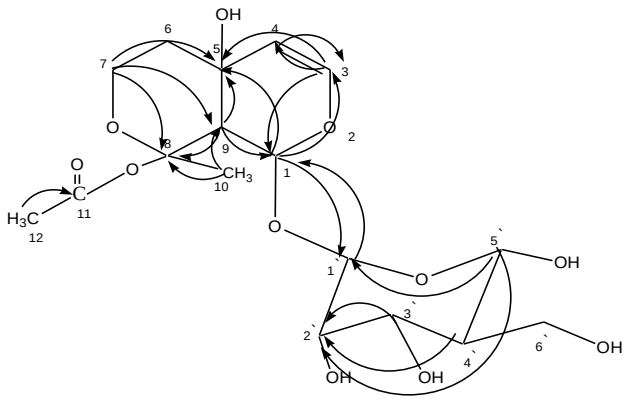


Figure 4.34 HMBC correlations of 8.

Table 4.9 ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data (600 MHz, CD_3OD) of 8.

No	δ^{1}H NMR (600 MHz, CD_3OD)	δ^{13}C NMR (600 MHz, CD_3OD)	DEPT (600 MHz, CD_3OD)	HMBC
1	6.04 (1H, d, H-1)	93.2	-CH-	142.6, 98.1, 72
2	-		-O-	-
3	6.36 (1H, d, *J* = 6.35, H-3)	142.6	-CH-	105.5, 93.2, 72
4	4.89 (hidden under OH signal)	105.5	-CH-	142.6
5	-	72	Q	-
6	1.27 (2H, t, H-6)	29.5	$-CH_2-$	-
7	2.10 (2H, t, H-7)	44.8	$-CH_2-$	87.4, 72, 54.1
8	-	87.4	Q	172.1
9	2.81 (1H, s, H-9)	54.1	-CH-	-
10	1.37 (3H, s, H-10)	20.9	CH_3-	87.4, 54.1, 44.8
11	-	172.1	C=O	-
12	1.94 (3H, s, H-12)	21.2	$-OCH_3$	93.2, 87.4, 72
1`	4.56 (1H, d, *J* =7.84, H-1`)	98.1	-CH-	93.2
2`	3.70 (1H, t, H-2`)	76.3	-CH-	-
3`	3.34 (1H, t, H-3`)	70.9	-CH-	76.9
4`	3.26(1H, m, H-4`)	76.9	-CH-	76.3
5`	3.17 (1H, t, H-5`)	73.8	-CH-	98.1, 76.3
6`	3.84 (2H, d, H-6`)	61.7	$-CH_2-$	-

Table 4.10 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) spectroscopic data comparison of 8 with ^{1}H and ^{13}C NMR (400 MHz, DMSO-d_6) of morroniside isolated from *Fructus corni.*

No	δ_H (600MHZ, CD_3OD) of 8	δ_C (600MHZ, CD_3OD) of 8	δ_H (400 MHz, DMSO-d_6) of morroniside	δ_C (400 MHz, DMSO-d_6) of morronisi de
1	6.04 (1H, d, H-1)	93.2	5.32 (1H, d, H-1)	94.6
2	-		-	-O-
3	6.36 (1H, d, *J* = 6.35, H-3)	142.6	7.40 (1H, s, H-2)	152.3
4	4.89 (hidden under OH signal)	105.5	-	109.2
5	-	72	1.85 (1H, m, H-5)	27.4
6	1.27 (2H, t, H-6)	29.5	1.85, 1.61 (each, 1H, m, H-6)	34.7
7	2.10 (2H, t, H-7)	44.8	5.32 (1H, m, H-7)	94.6
8	-	87.4	2.50 (1H, m, H-8)	39.5
9	2.81 (1H, s, H-9)	54.1	3.74 (3H, s, OCH_3-9)	52.0
10	1.37 (3H, s, H-10)	20.9	1.20 (3H, d, H-10)	20.4
11	-	172.1	-	166.2
12	1.94 (3H, s, H-12)	21.2	3.74 (1H, m, H-12)	63.3
1`	4.56 (1H, d, *J* =7.84, H-1`)	98.1	4.93 (1H,d, H-1`)	98.1
2`	3.70 (1H, t, H-2`)	76.3	-	-
3`	3.34 (1H, t, H-3`)	70.9	-	-
4`	3.26(1H, m, H-4`)	76.9	-	-
5`	3.17 (1H, t, H-5`)	73.8	-	-
6`	3.84 (2H, d, H-6`)	61.7	-	-

Table 4.11 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) data comparison of 8 with ^{1}H and ^{13}C NMR (D_2O) of 6-epi-8-acetylharpagide isolated from *Caryopteris x clandonensis*.

No	δ_H (600MHZ, CD_3OD) of 8	δ_C (600MHZ, CD_3OD) of 8	δ_H (D_2O) of 6-epi-8-acetylharpagide	δ_C (D_2O) of 6-epi-8-acetylharpagide
1	6.04 (1H, d, H-1)	93.2	5.95 s	95.8
2	-		-	
3	6.36 (1H, d, *J* = 6.35, H-3)	142.6	6.51 dd(6.4, 1.0)	145.3
4	4.89 (hidden under OH signal)	105.5	5.11 d (6.5) -	104.0 -
5	-	72		74.6
6	1.27 (2H, t, H-6)	29.5	4.27 dd (12.5, 6.6)	77.8
7	2.10 (2H, t, H-7)	44.8	1.57 dd (13.5, 12.5) 2.24 dd (13.5, 6.6)	46.1 -
8	-	87.4	-	86.6
9	2.81 (1H, s, H-9)	54.1	2.70 s	56.7
10	1.37 (3H, s, H-10)	20.9	1.44 s	23.5
11	-	172.1	-	176.8
12	1.94 (3H, s, H-12)	21.2	2.03 s	24.3
1`	4.56 (1H, d, *J* =7.84, H-1`)	98.1	4.71 dd (8.0, 1.0)	100.1
2`	3.70 (1H, t, H-2`)	76.3	3.25 ddd(9.3, 8.2, 1.1)	75.2
3`	3.34 (1H, t, H-3`)	70.9	3.44 – 3.50	78.1
4`	3.26(1H, m, H-4`)	76.9	3.38 dd (9.9, 1.0)	72.3
5`	3.17 (1H, t, H-5`)	73.8	3.44 – 3.50	79.0
6`	3.84 (2H, d, H-6`)	61.7	3.71 dd (12.4, 5.6) 3.91 d (12.4)	63.3

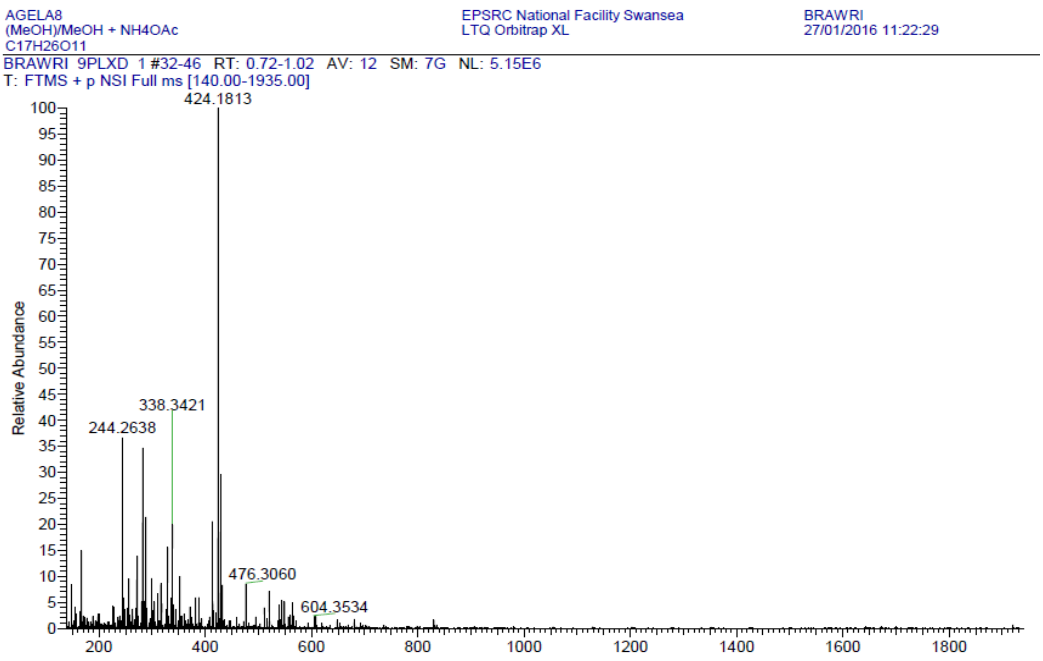


Figure 4.35 An accurate mass spectrum of 8.

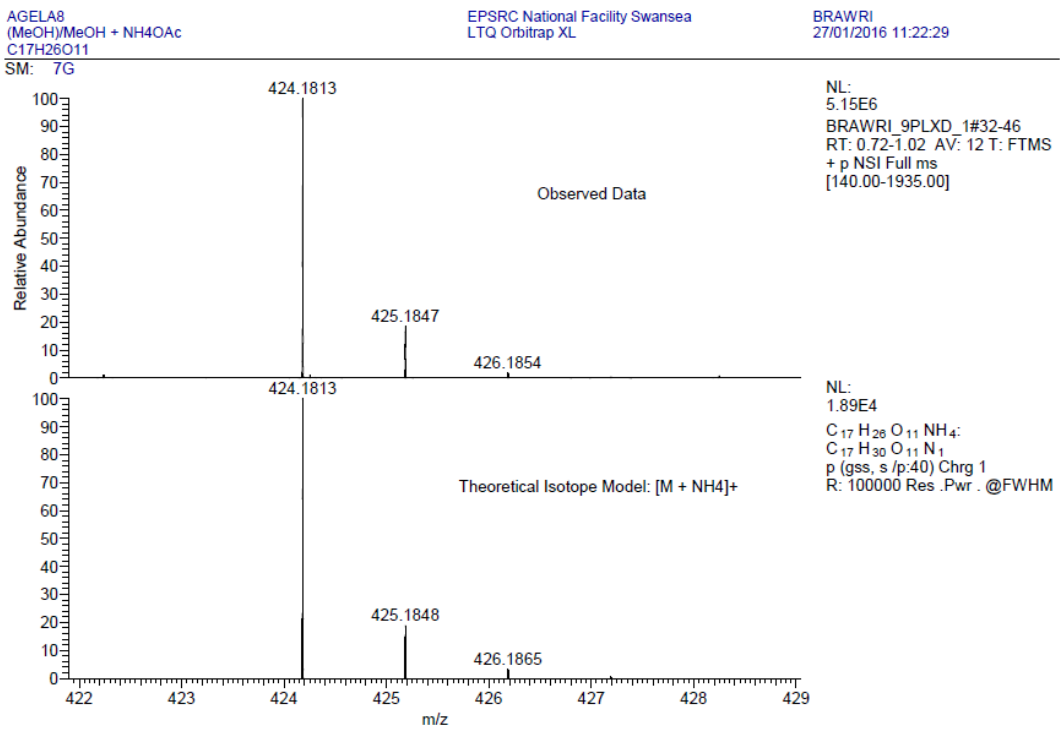


Figure 4.35 Contaniued.

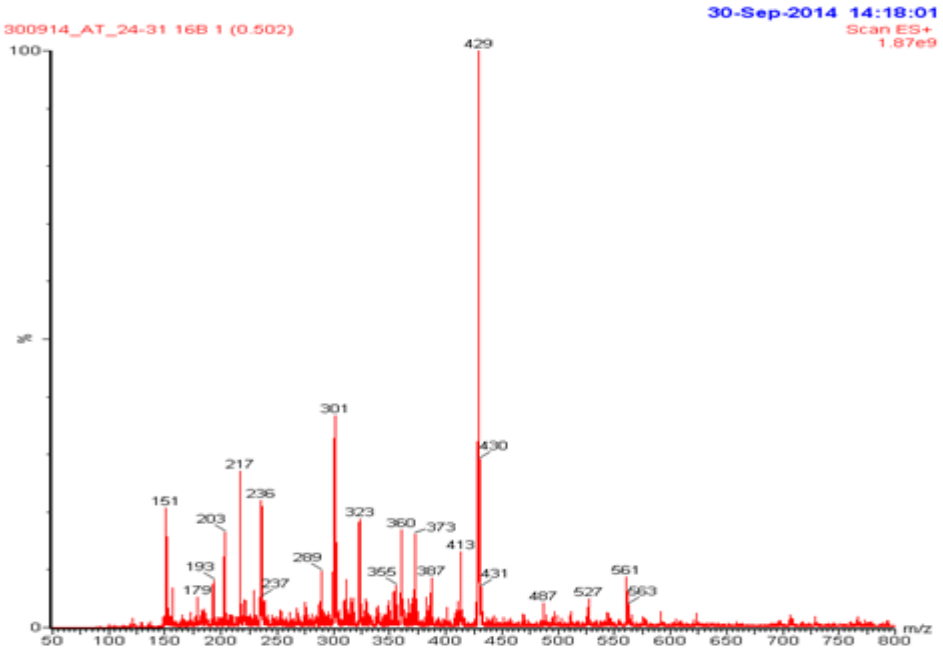


Figure 4.36 ES+ mass spectrum of 8.

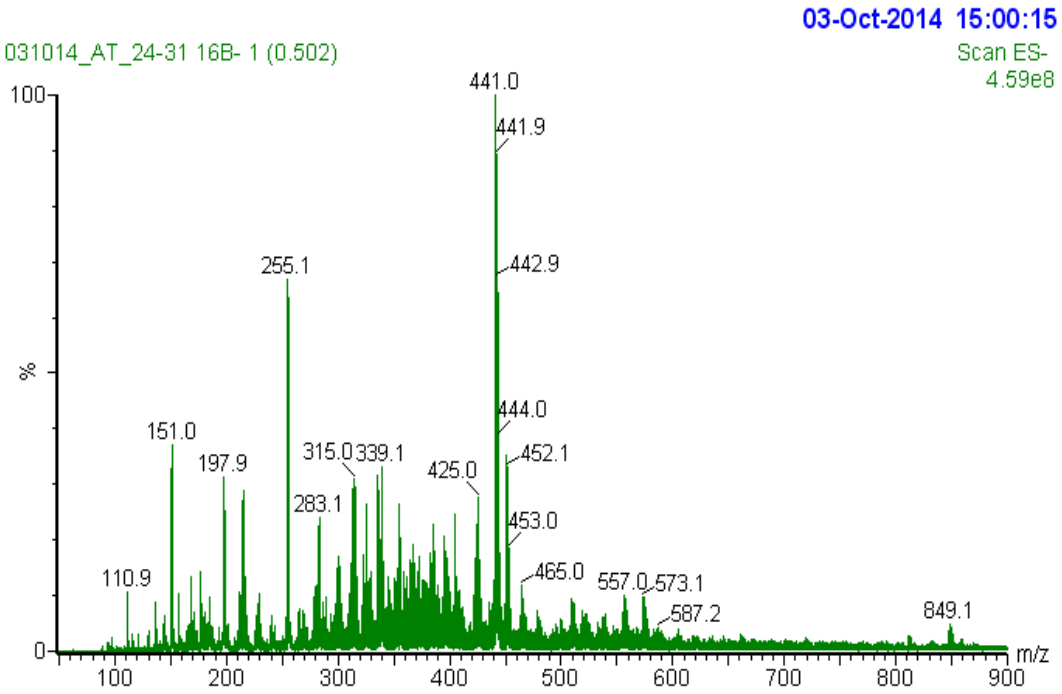


Figure 4.37 ES- mass spectrum of 8.

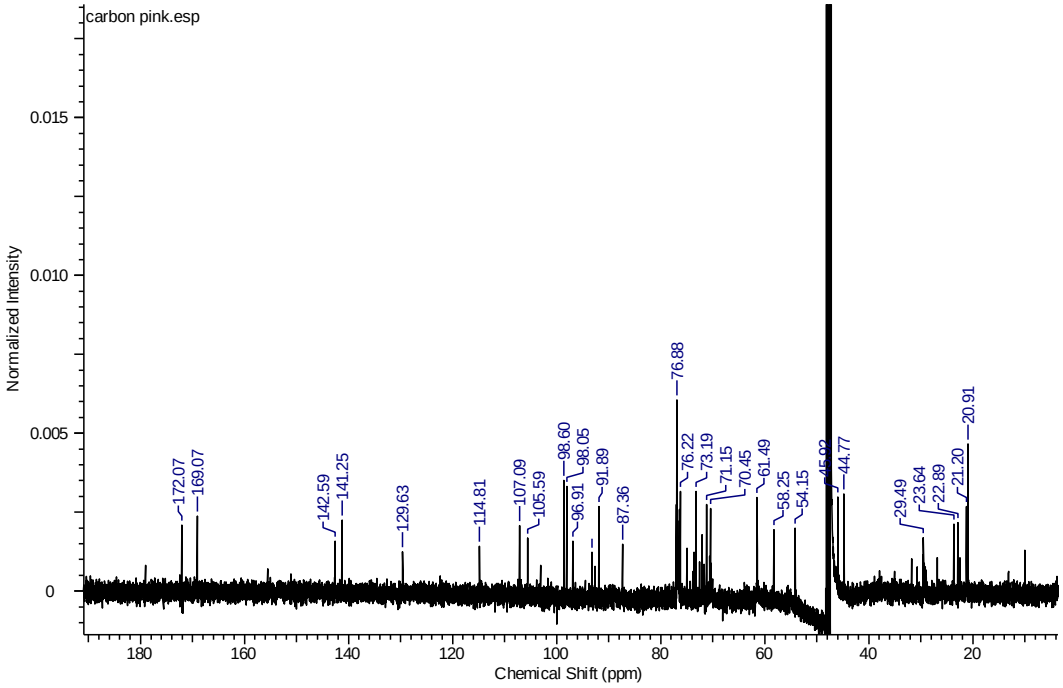


Figure 4.38 ^{13}CNMR spectrum (600 MHz, CD_3OD) of 8.

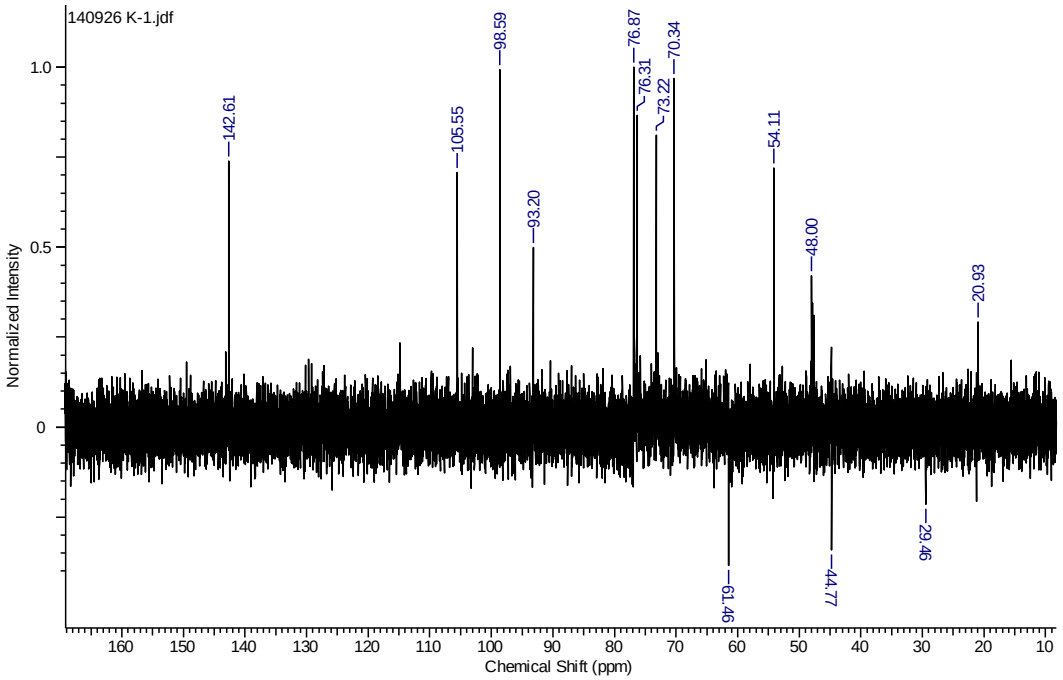


Figure 4.39 DEPT spectrum (600 MHz, CD_3OD) of 8.

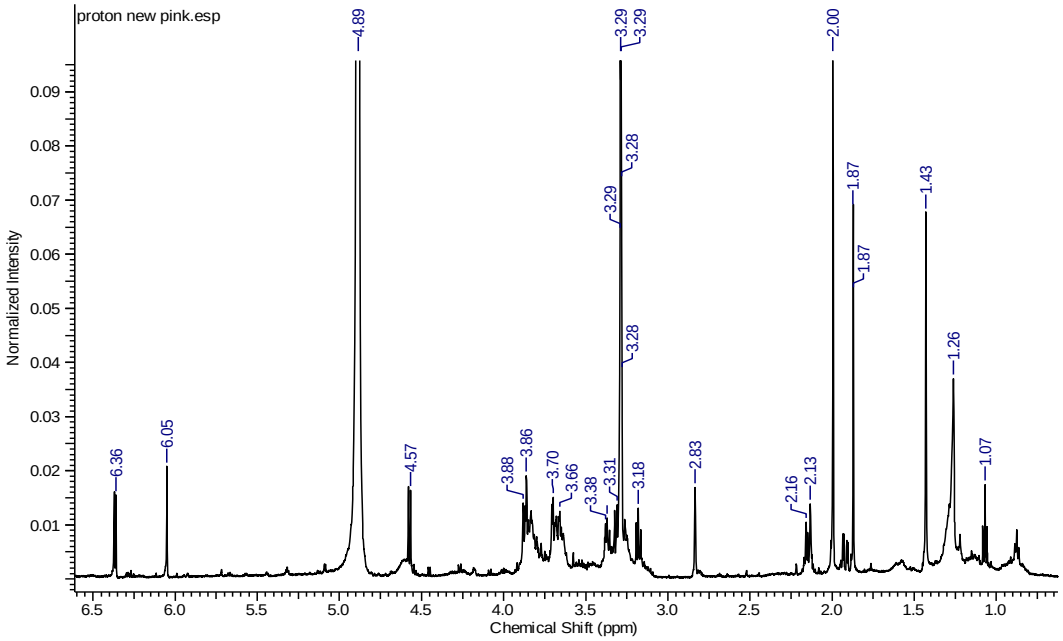


Figure 4.40 ^{1}HNMR spectrum (600 MHz, CD_3OD) of 8.

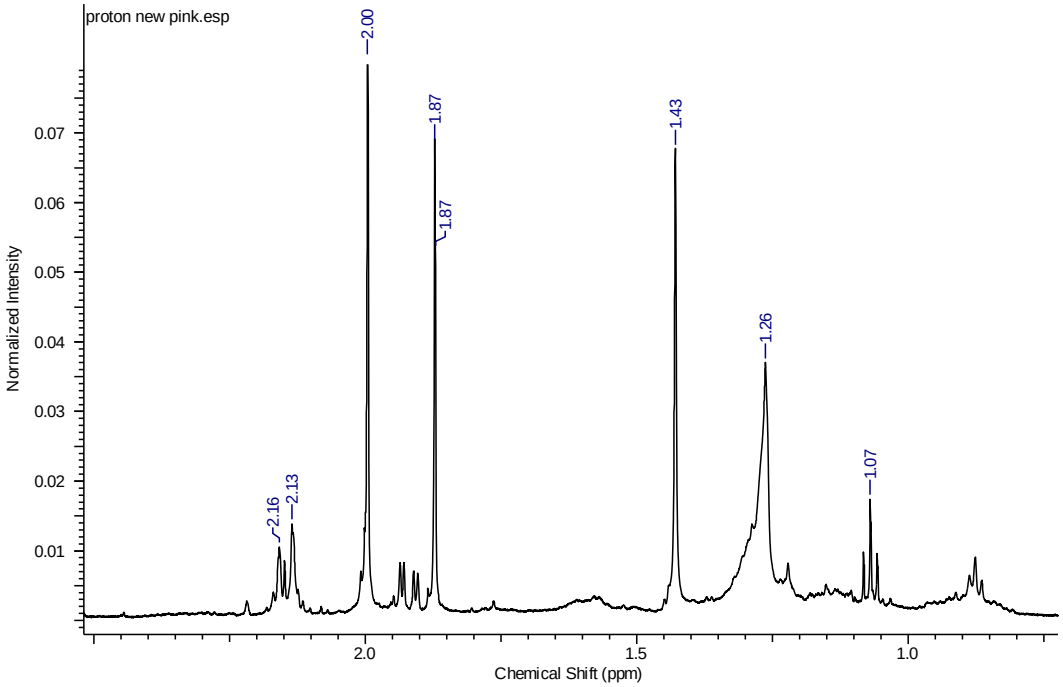


Figure 4.41 Expansion of ^{1}HNMR spectrum (600 MHz, CD_3OD) of 8.

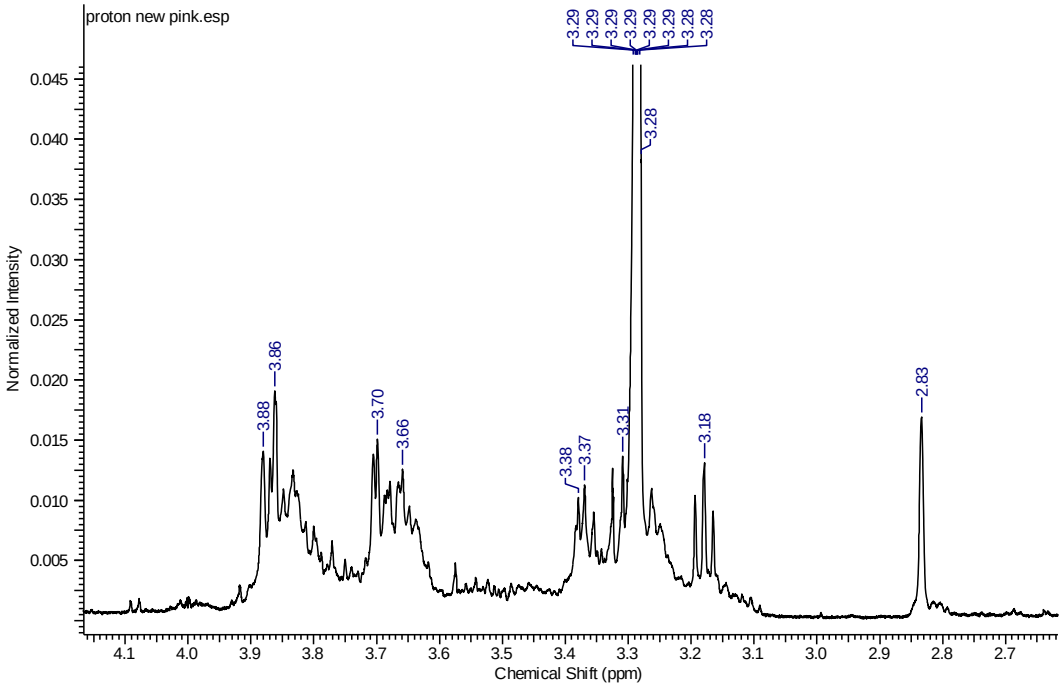


Figure 4.41 Continued.

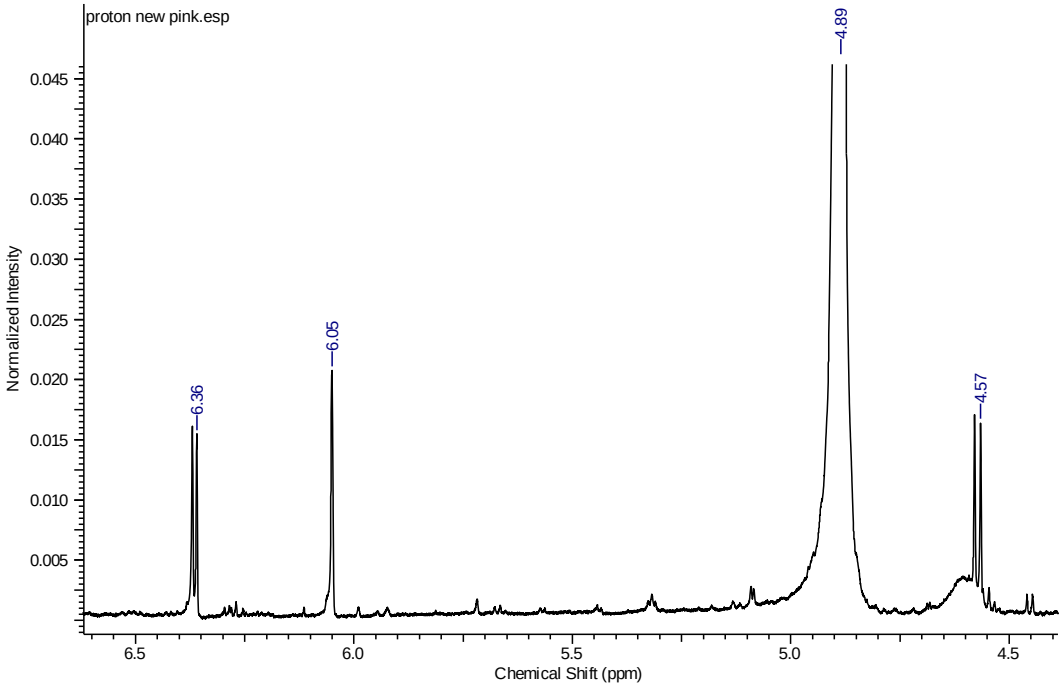


Figure 4.41 Continued.

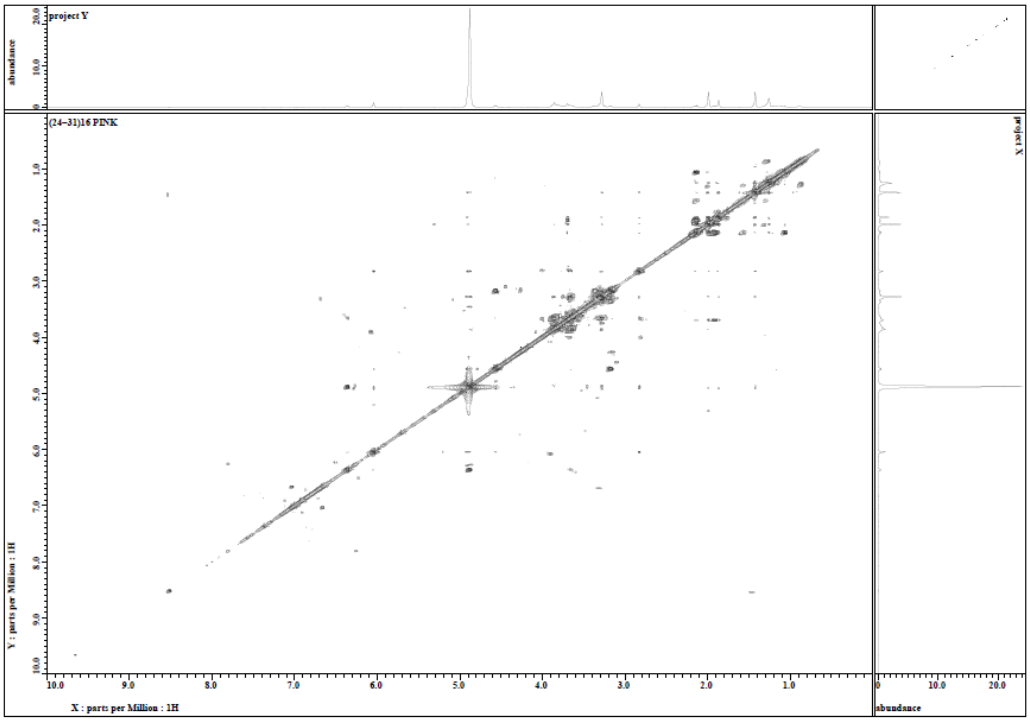


Figure 4.42 COSY NMR spectrum (600 MHz, CD_3OD) of 8.

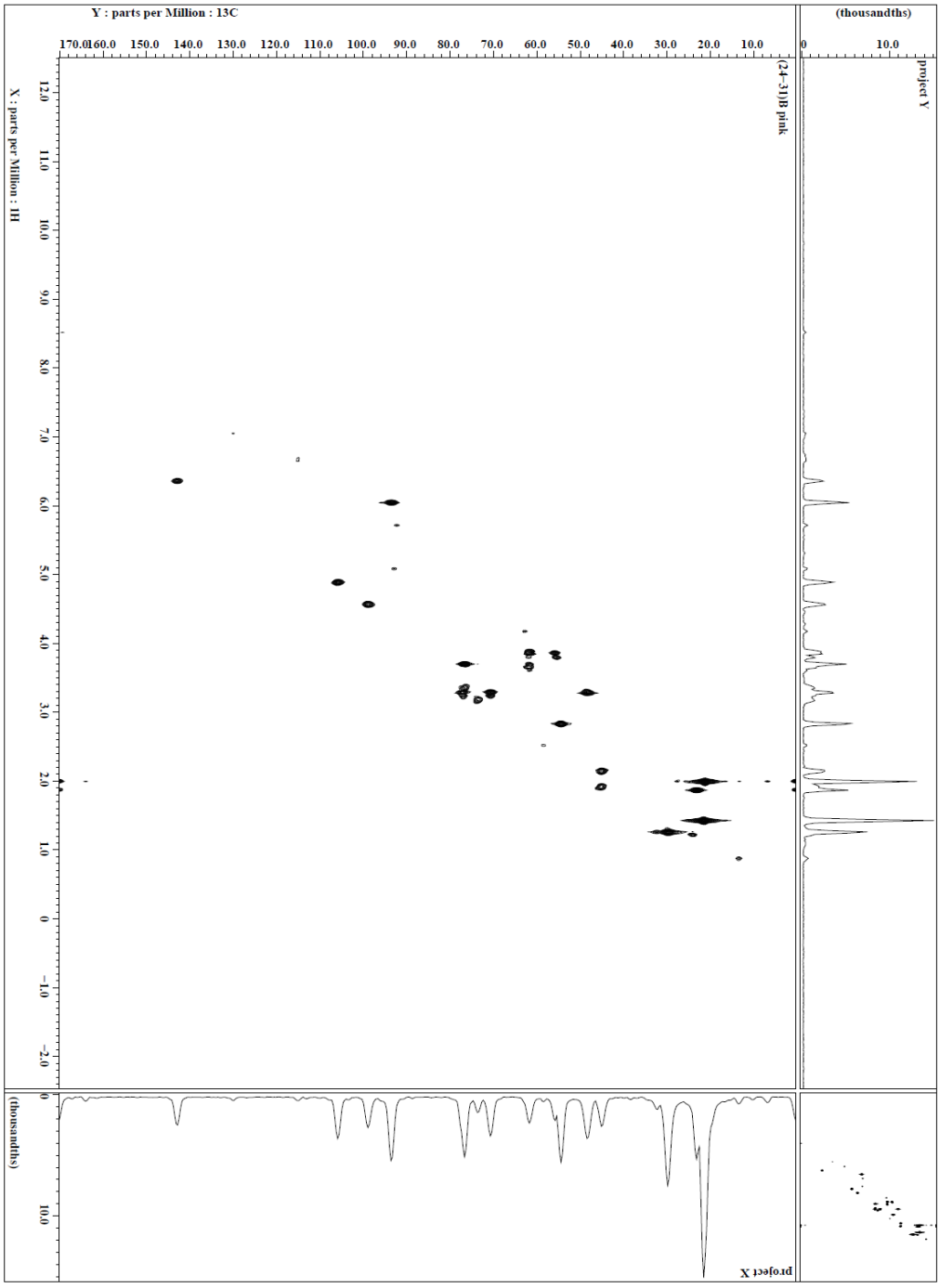


Figure 4.43 HMQC spectrum (600 MHz, CD_3OD) of 8.

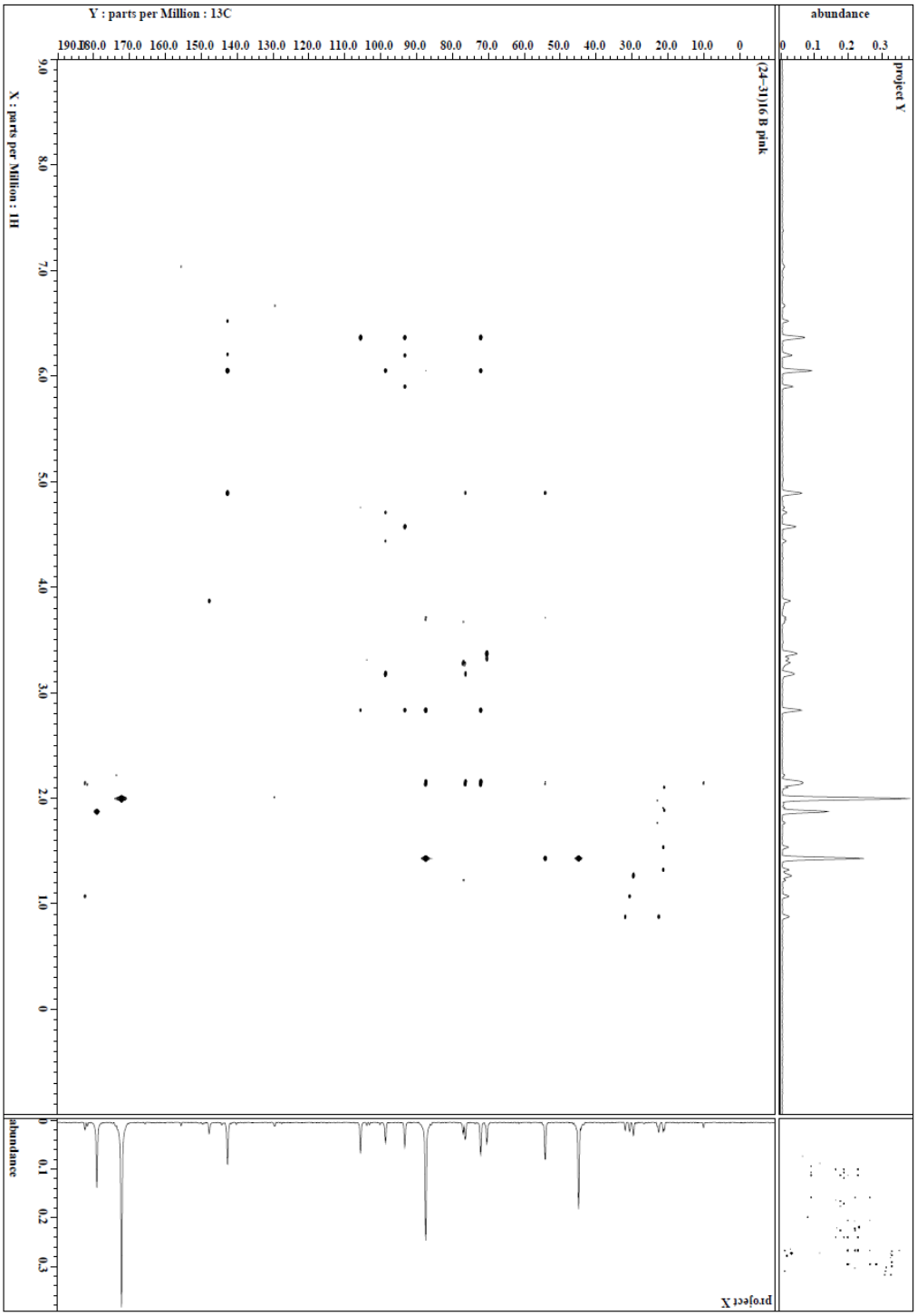
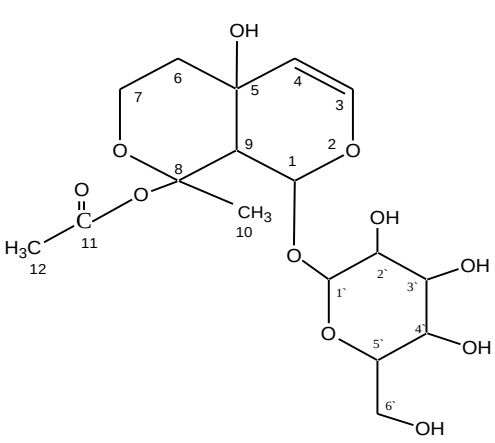
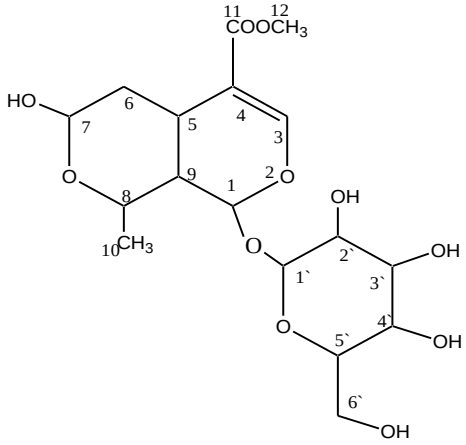


Figure 4.44 HMBC spectrum (600 MHz, CD_3OD) of 8.

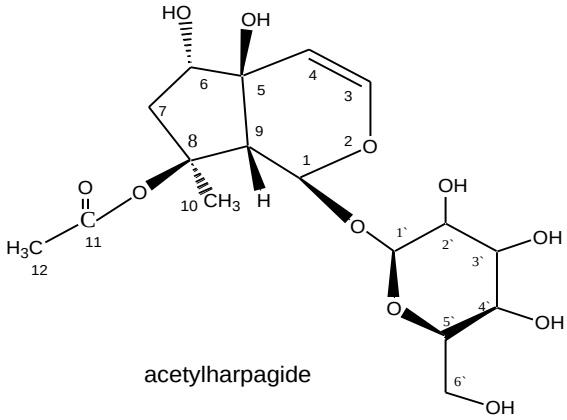
From the best of my knowledge compound **8** isolated from *Teucrium davaeanum* appears to be a novel glycoside for which the name davaeanuside B is proposed. It has comparable ^{1}H and ^{13}C NMR data and the same molecular weight of morroniside (m/z = 406.1) isolated from *Fructus corni* but they are different in chemical structure (Lui *et al*., 2009) . **8** appears to be an isomer of the iridoid glycoside named acetylharpagide isolated from *Caryopteris x clandonensis*. Table 4.10 and Table 4.11 show a comparison of ^{1}H and ^{13}C NMR data of **8** with ^{1}H and ^{13}C NMR data of morroniside and acetylharpagide. The stereochemistry of **8** has not been confirmed; it could be achieved via X-ray crystallography method and with the help of nuclear overhauser effect NMR spectroscopy (NOE). Figure 4.45 shows the structures of compound 8, morroniside and 6-epi-8-acetylharpagide. Iridoid glycosides are common in many genera of the Lamiaceae*: Prostantheroideae, Ajugoideae, Prasioideae, Scutellarioideae, Stachydoideae-Marrubieae* and *St.-Stachydeae* (Kooiman, 1972), as well as in species of *Teucrium*: *Teucrium polium* (Jaradat, 2015), (Rizk *et al*., 1986), *Teucrium arduini* (Ruhdorfer and Rimpler, 1981), (Çakir *et al.*, 2006).



Compound-8



Morroniside



acetylharpagide

Figure 4.45 Structure of 8, morroniside and 6-epi-8-acetylharpagide.

4.2.4 Procedure for isolation compounds 9 & 10

The results of TLC analysis of fractions (35-36) Table 4.485 mg (section 2.5.5) showed that it composed mainly from two bands (compound **9** & **10**), therefore, preparative TLC plates was carried out to purify these two compounds (section 2.5.3). 12 plates were used; the bands corresponding to two compounds were collected in small vials using a spatula, then dissolved in methanol, filtered by filter paper to remove the silica gel then dried under nitrogen gas. Compound **9** was 16 mg while **10** was 25 mg.

Fractions (39-40) Table 4.4 755 mg were also contained the compound **10** therefore, they were further purified using reverse phase silica gel column (40 × 3.5 cm) (secton 2.5.2). The fractionation process illustrated in Table 4.12.

Table 4.12 Process to purify a further quantity of compound 10.

Mobile phase	**Composition**	**Volume (ml)**	**Fr. number**	**Combined the fraction**
$CHCl_3$	100	100	1	(1-16) discarded
$CHCl_3$:MeOH	90:10	100	2-6	(17-19) compound **6** pure
$CHCl_3$:MeOH	70:30	100	7-26	20-25
$CHCl_3$:MeOH	60:40	100	27-34	26-28
$CHCl_3$:MeOH	95:5	200	35-48	29-30
MeOH:H_2O	90:10	200	49-64	31-40
MeOH:H_2O	80:20	200	65-70	41-43
MeOH:H_2O	70:30	200	71-79	44-79

Sub-fraction (41-43) Table 4.12 30 mg contained the compound **10**, so it was further purified on (25g) reverse phase silica gel column (30 × 2.5 cm), the silica gel was mixed with methanol and the fractionation was carried out with the methanol, 17 fractions (5 ml each) were collected. Sub-fractions (10-18) 15 (mg) was in pure form (compound **10**).

4.2.4.1 Structure elucidation of compound 9 (sucrose).

Compound **9** was isolated as a white solid substance from the crude glycosides extract of *T. davaeanum*. TLC analysis of **9** on silica gel showed no UV absorbtion at 254nm or 375 nm and a dark green colour spot (Rf = 0.15) after vanillin/sulphuric acid spraying Figure 4.75. Compound **9** has the molecular formula $C_{12}H_{22}O_{11}$ (m/z = 342.1240) as determined by an accurate mass measurement, the measured accurate mass was 343.1240 and the calculated accurate mass was 343.1235 corresponding to the molecular ion peak $[M + H]^+$ Figure 4.47. The Accurate mass measurement was carried out at the EPSRC UK National Mass Spectrometry Facility at Swansea University Figure 4.48 is shown expansion of accurate mass spectrum of **9**. The ES+ mass spectrum of **9** figure Figure 4.49 displayed a peak at m/z = 365 $[M + Na]^+$ suggesting a molecular weight of 342. While the ES- mass spectrum Figure 4.50 showed a peak at m/z = 341 $[M – H]^-$.

The ^{1}H NMR, ^{13}C NMR and HMBC data of **9** are summarized in Table 4.13. The ^{13}C NMR (600 MHz, CD_3OD) spectrum Figure 4.51 showed 12 signals, indicates the molecule has 12 carbon atoms. The glucose and fructose anomeric carbons appears at δ values of 93 C-1 and 62.1 C-1`, Figure 4.52 expansion of $^{13}$CNMR spectrum of **9**. The DEPT spectrum of **9** Figure 4.53 showed the presence of 3 ($-CH_2-$) groups, one of glucose at δ 60.8 C-6 and two of fructose at δ 62.1 C-1` and 62.6 C-6`.

The ^{1}H NMR (600 MHz, CD_3OD) spectrum of **9** Figure 4.54 showed signals in the region between δ 3.5 and δ 4.5 which is characteristic for a sugar molecule. Furthermore, the signal at δ 5.38 (1H, d, *J* = 3.78, H-1) is corresponding to glucose anomeric proton. While the signal at δ 3.82 (1H, d, H-1`) is corresponding to fructose anomeric proton, Figure 4.55 is shown expansion of ^{1}HNMR spectrum of **9**. Figure 4.56 is illustrated HMQC spectrum of **9**.

In HMBC of **9**

Figure 4.58 cross peaks from the anomeric proton of glucose [δ5.38 (1H, d, *J* = 3.78, H-1) to the carbon atoms C-2 δ 73.4 and C-3 δ 72.2 of glucose and C-2` δ 104 of fructose were observed. Table 4.14 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) spectroscopic data comparison of **9** with ^{1}H and ^{13}C NMR (500 MHz, H_2O) of sucrose used in journal articles (Duker and Serianni, 1993). Figure 4.45 is shown HMBC correlations of **9**.

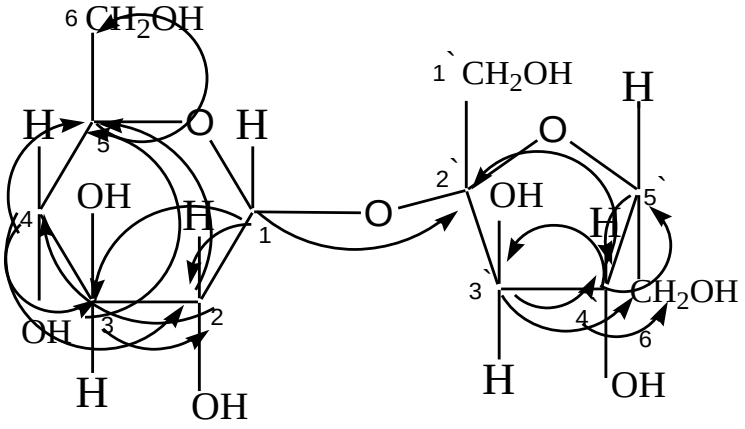


Figure 4.46 HMBC correlations of 9.

Table 4.13 ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data (600 MHz, CD_3OD) of 9.

No	^{1}HNMR (600 MHz, CD_3OD) of 9	^{13}CNMR (600 MHz, CD_3OD) of 9	DEPT	HMBC
1	5.38 (1H, d, *J* = 3.78, H-1)	93	CH	73.4, 72.2, 104
2	3.81 (1H, t, H-2)	73.4	CH	70, 73.6
3	3.42 (1H, d, *J* = 3.78, H-3)	72.2	CH	70, 73.4
4	3.69 (1H, t, H-4)	73.6	CH	70, 72.2, 73.4
5	3.33 (1H, t, H-5)	70	CH	60.8, 73.4
6	Overlapped	60.8	CH_2	-
1`	3.82 (1H, d, H-1`)	62.1	CH_2	-
2`	-	104	Q	-
3`	4.07 (1H, d, *J* = 8.24, H-3`)	78	CH	62.6, 74
4`	4.00 (1H, t, H-4`)	74	CH	62.6, 78, 82
5`	3.73 (Overlapped)	82	CH	74
6`	3.74 (Overlapped)	62.6	CH_2	74, 82, 104

Table 4.14 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) data comparison of 9 with ^{1}H and ^{13}C NMR (500 MHz, H_2O) of sucrose.

No	^{1}HNMR(600 MHz, CD_3OD) of 9	^{13}CNMR (600 MHz, CD_3OD) of 9	^{1}HNMR(500 MHz, H_2O) of sucrose (Duker and Serianni, 1993)	^{13}CNMR (500 MHz, H_2O) of sucrose (Duker and Serianni, 1993)
1	5.38 (1H, d, *J* = 3.78, H-1)	93	5.49	94.5
2	3.81 (1H, t, H-2)	73.4	3.63	73.4
3	3.42 (1H, d, *J* = 3.78, H-3)	72.2	3.83	74.9
4	3.69 (1H, t, H-4)	73.6	3.54	71.5
5	3.33 (1H, t, H-5)	70	3.88-3.93	74.7
6	Overlapped	60.8	3.88-3.93	62.4
1`	-	62.1	3.75	63.6
2`	3.82 (1H, d, H-1`)	104	-	106.0
3`	4.07 (1H, d, *J* = 8.24, H-3`)	78	4.29	78.7
4`	4.00 (1H, t, H-4`)	74	4.12	76.3
5`	3.73 (Overlapped)	82	3.96	83.7
6`	3.74 (Overlapped)	62.6	3.88-3.93	64.7

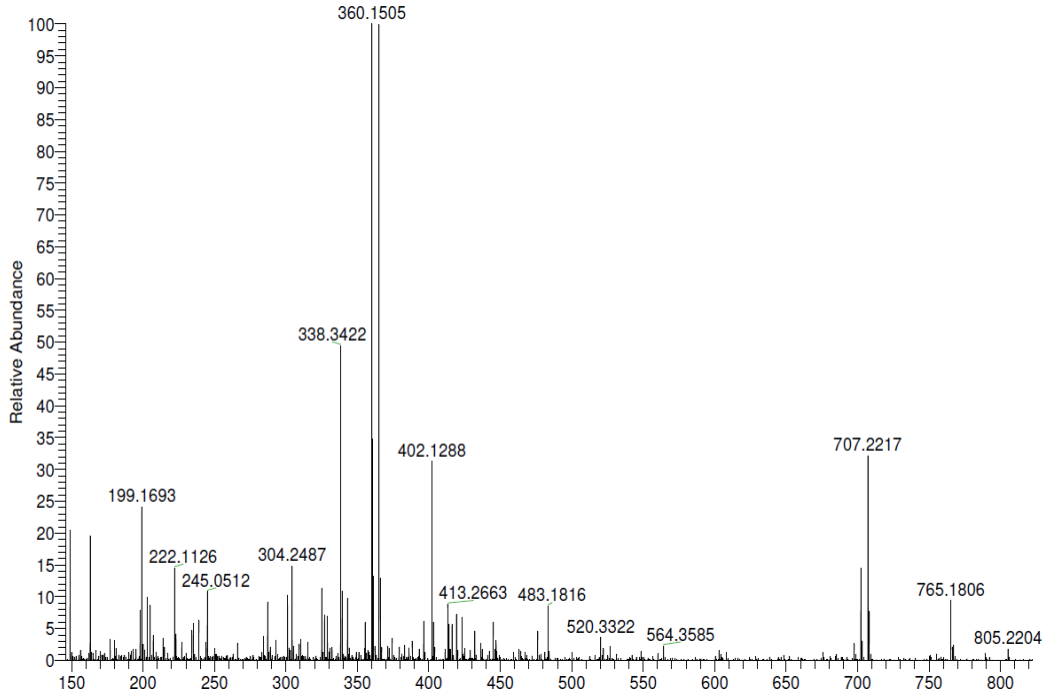


Figure 4.47 Accurate mass spectrum of 9.

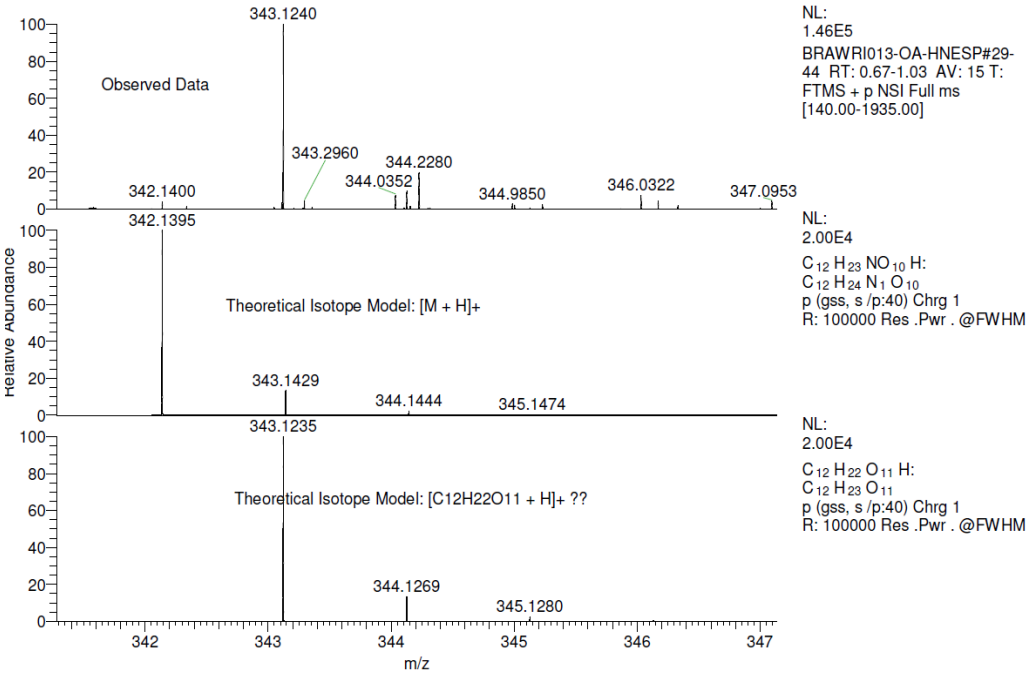


Figure 4.48 Expansion of accurate mass spectrum of 9.

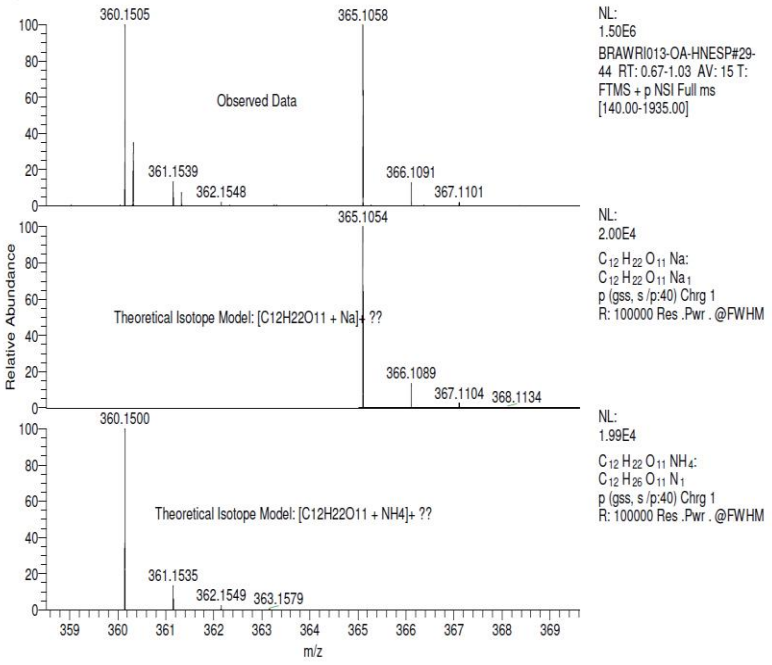


Figure 4.48 Continued.

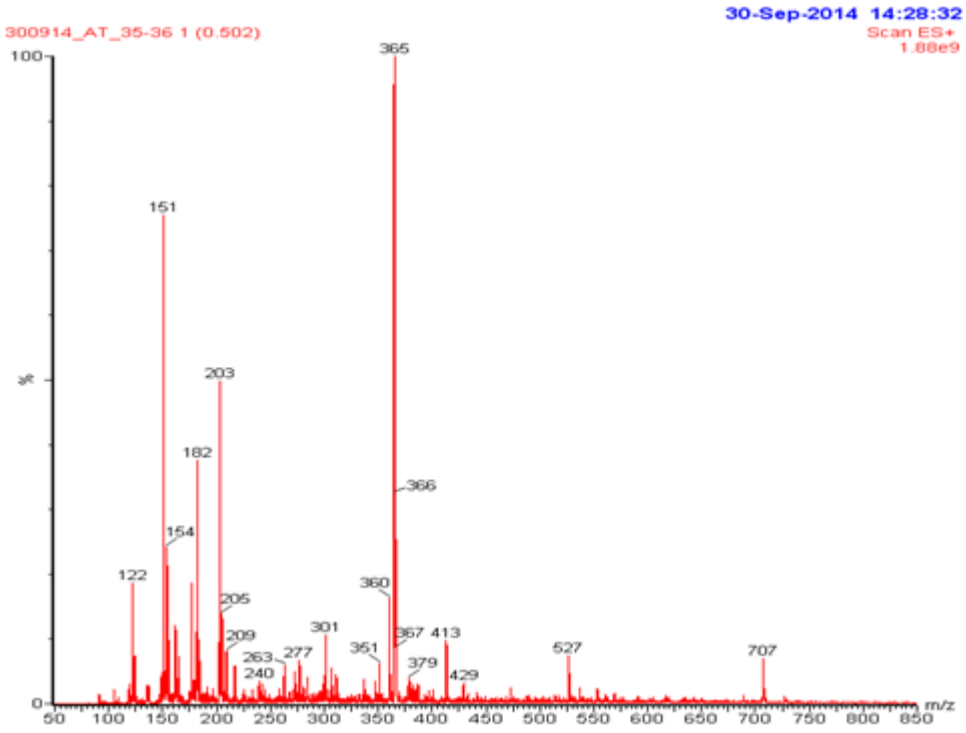


Figure 4.49 ES+ mass spectrum of 9.

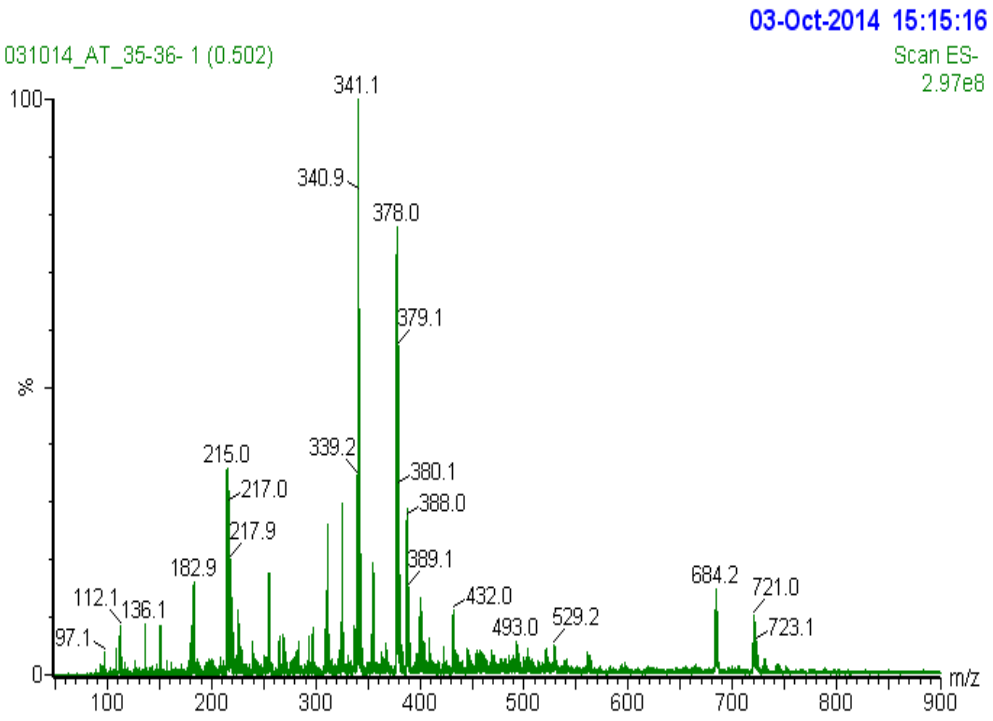


Figure 4.50 ES- mass spectrum of 9.

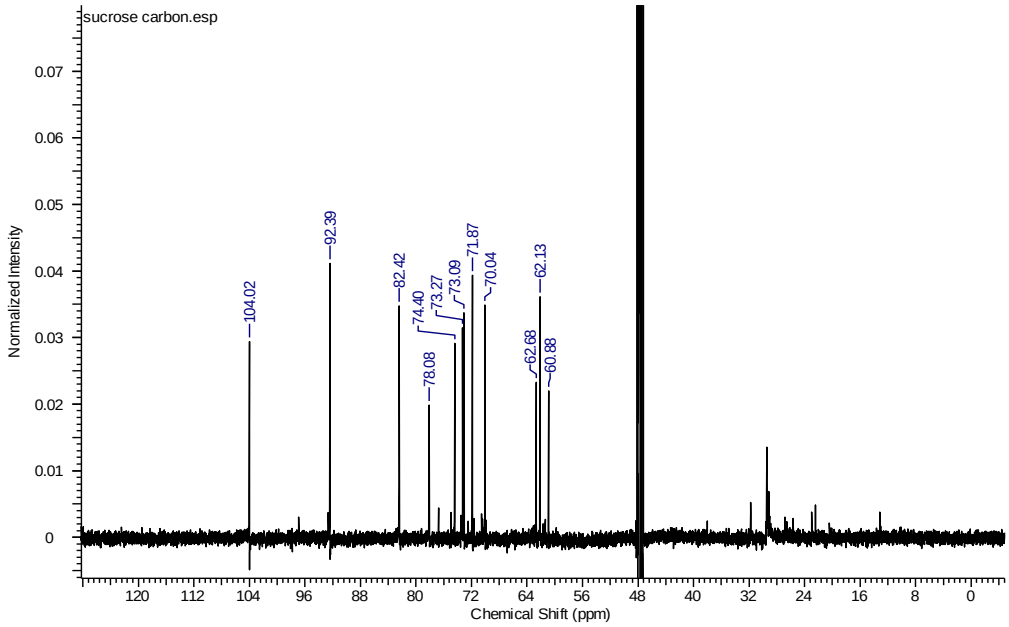


Figure 4.51 ^{13}C NMR spectrum of 9 (600 MHz, CD_3OD).

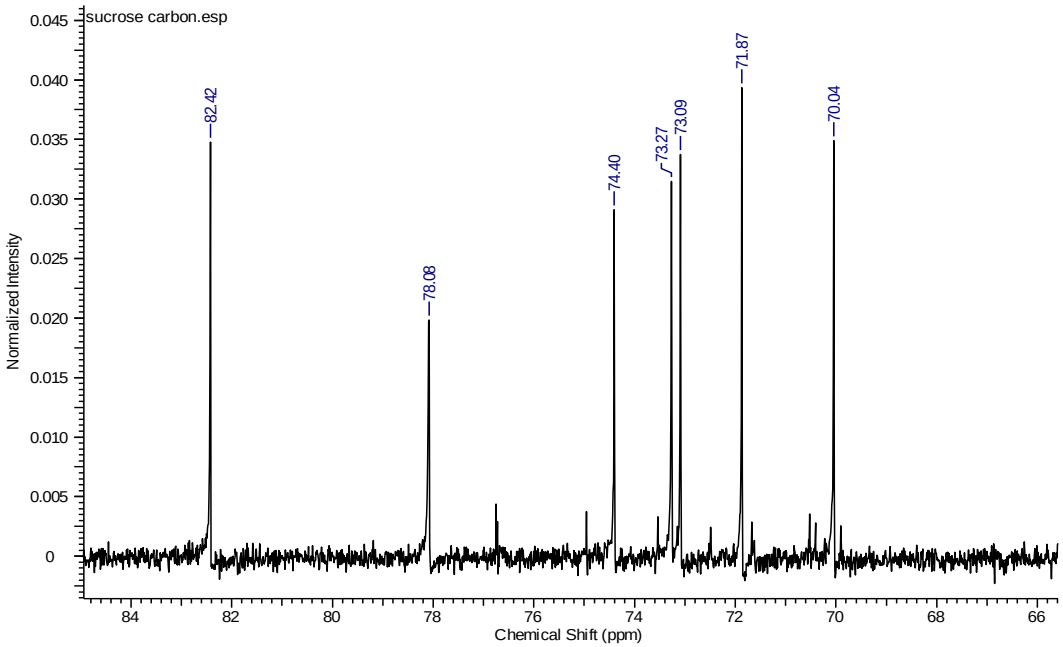


Figure 4.52 Expansion of ^{13}CNMR spectrum (600 MHz, CD_3OD) of 9.

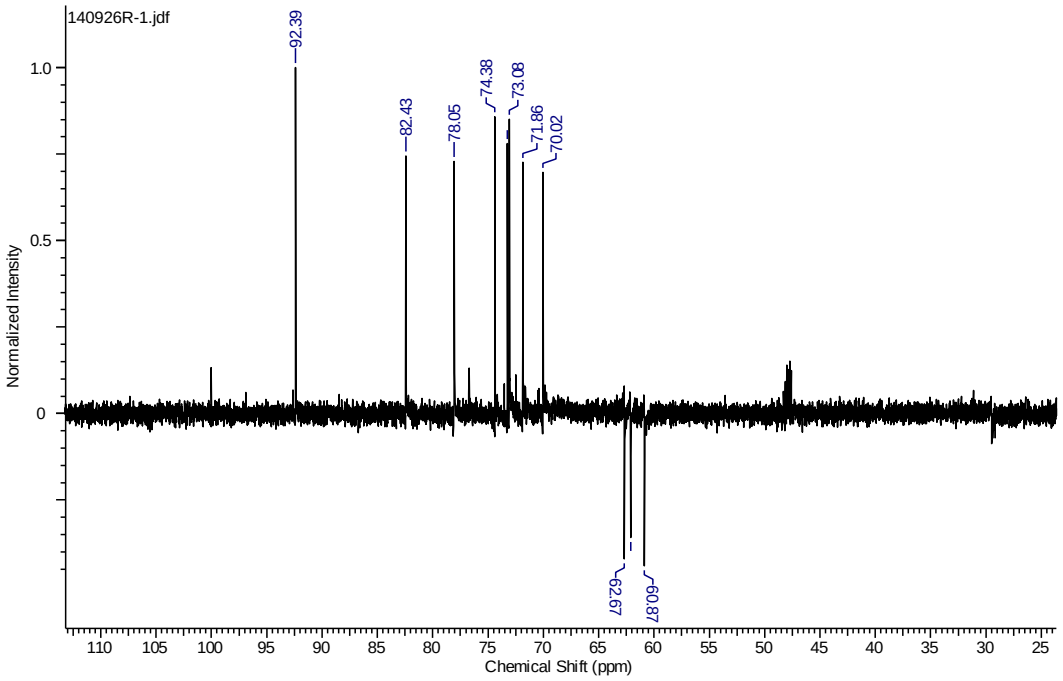


Figure 4.53 DEPT spectrum (600 MHz, CD_3OD) of 9.

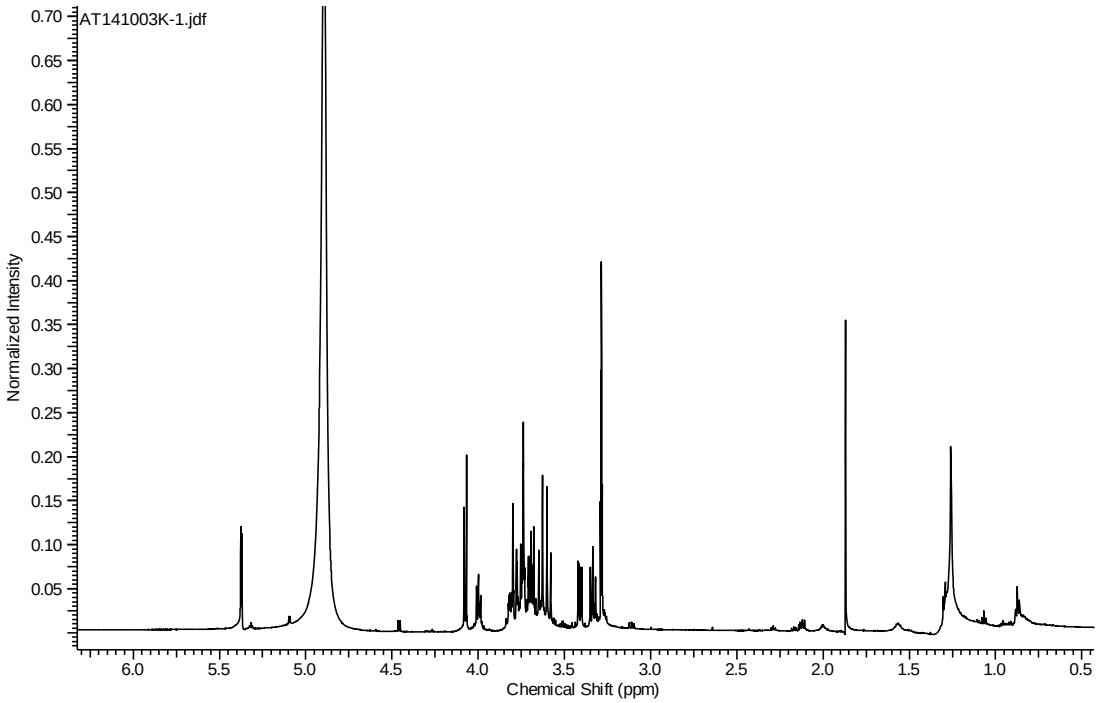


Figure 4.54 ^{1}H NMR spectrum (600 MHz, CD_3OD) of 9.

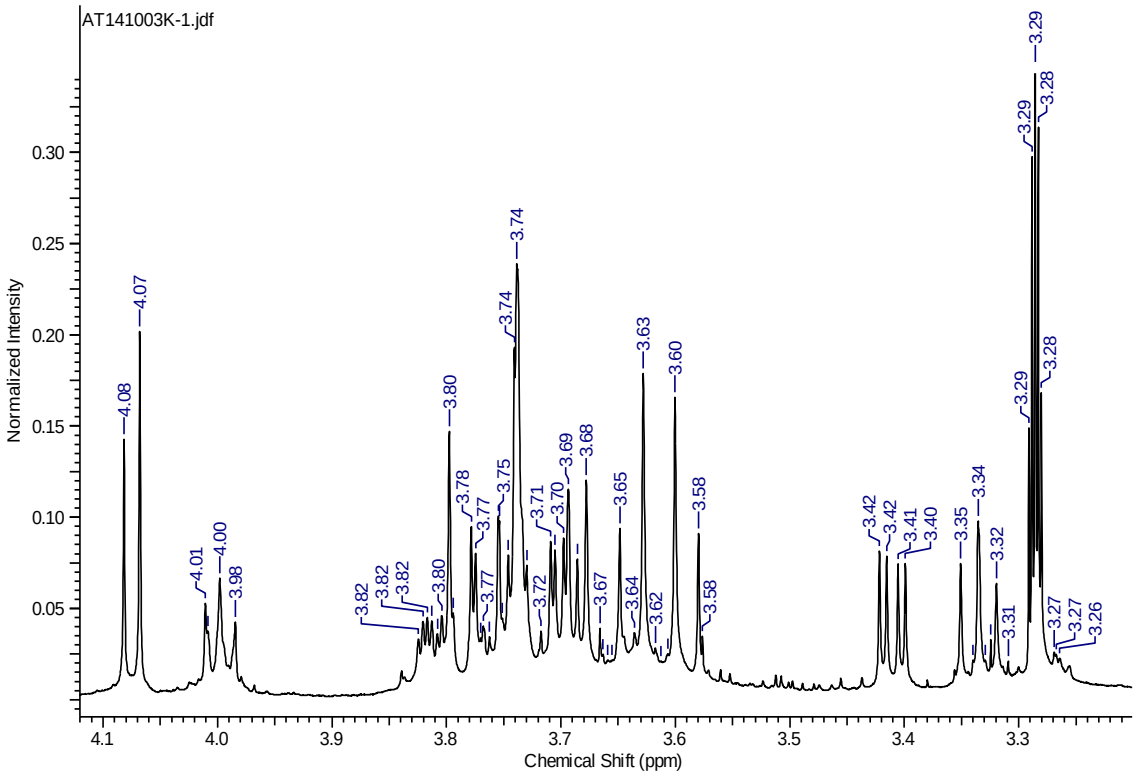


Figure 4.55 Expansion of ^{1}H NMR spectrum (600 MHz, CD_3OD) of 9.

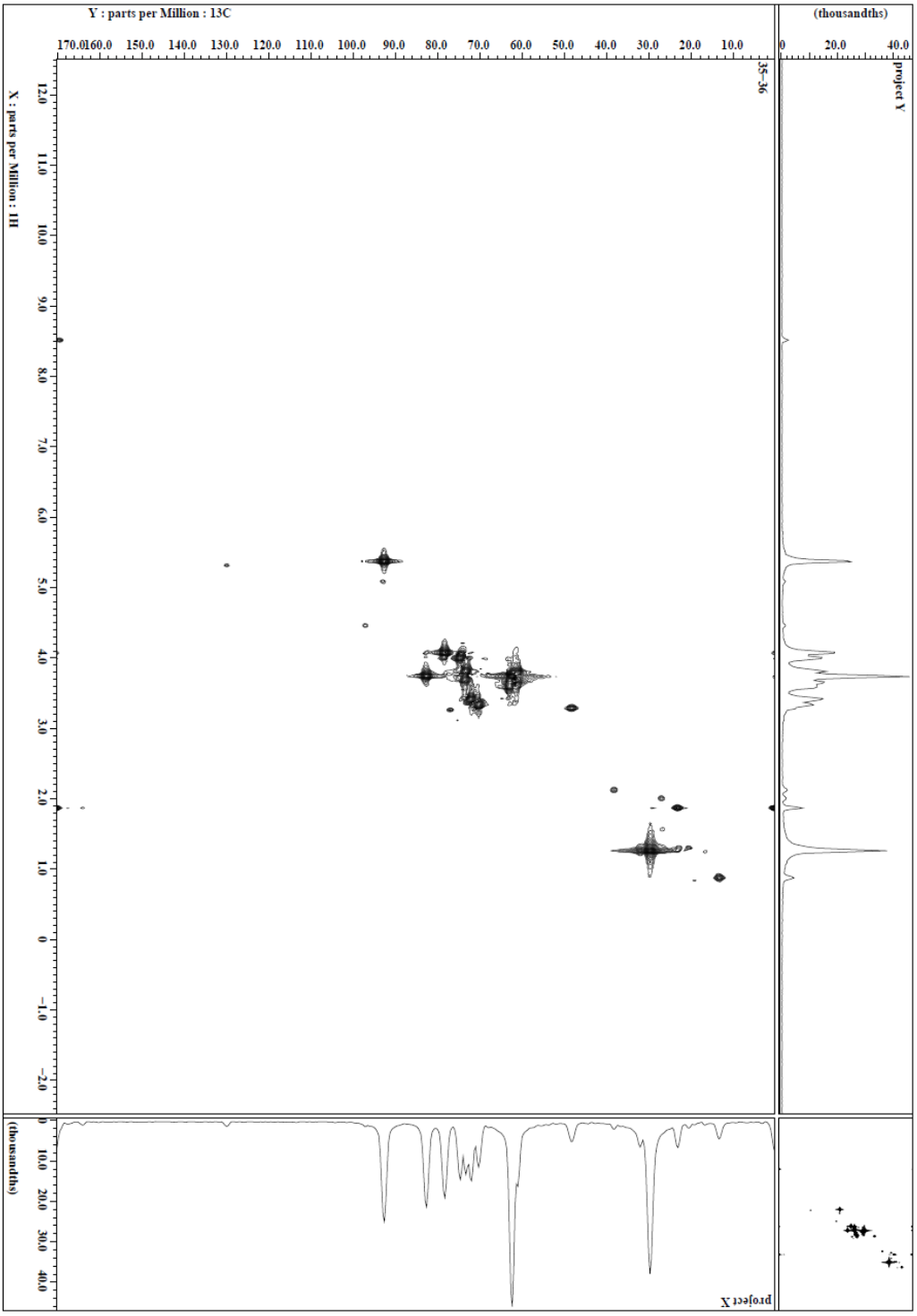


Figure 4.56 HMQC spectrum (600 MHz, CD_3OD) of 9.

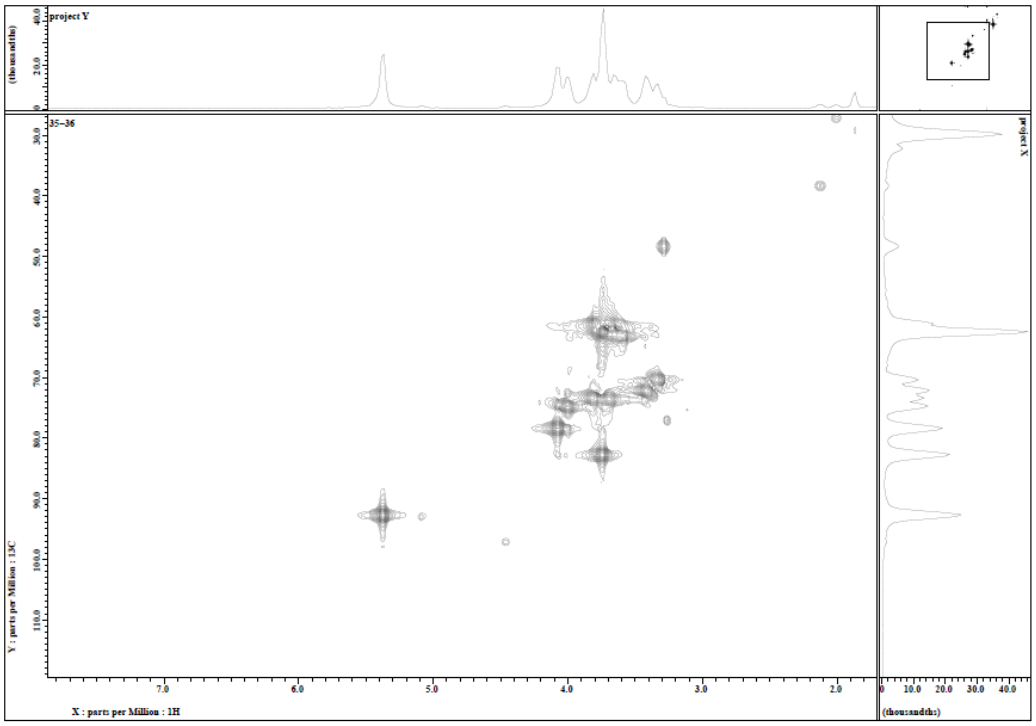


Figure 4.57 Expansion of HMQC spectrum (600 MHz, CD_3OD) of 9.

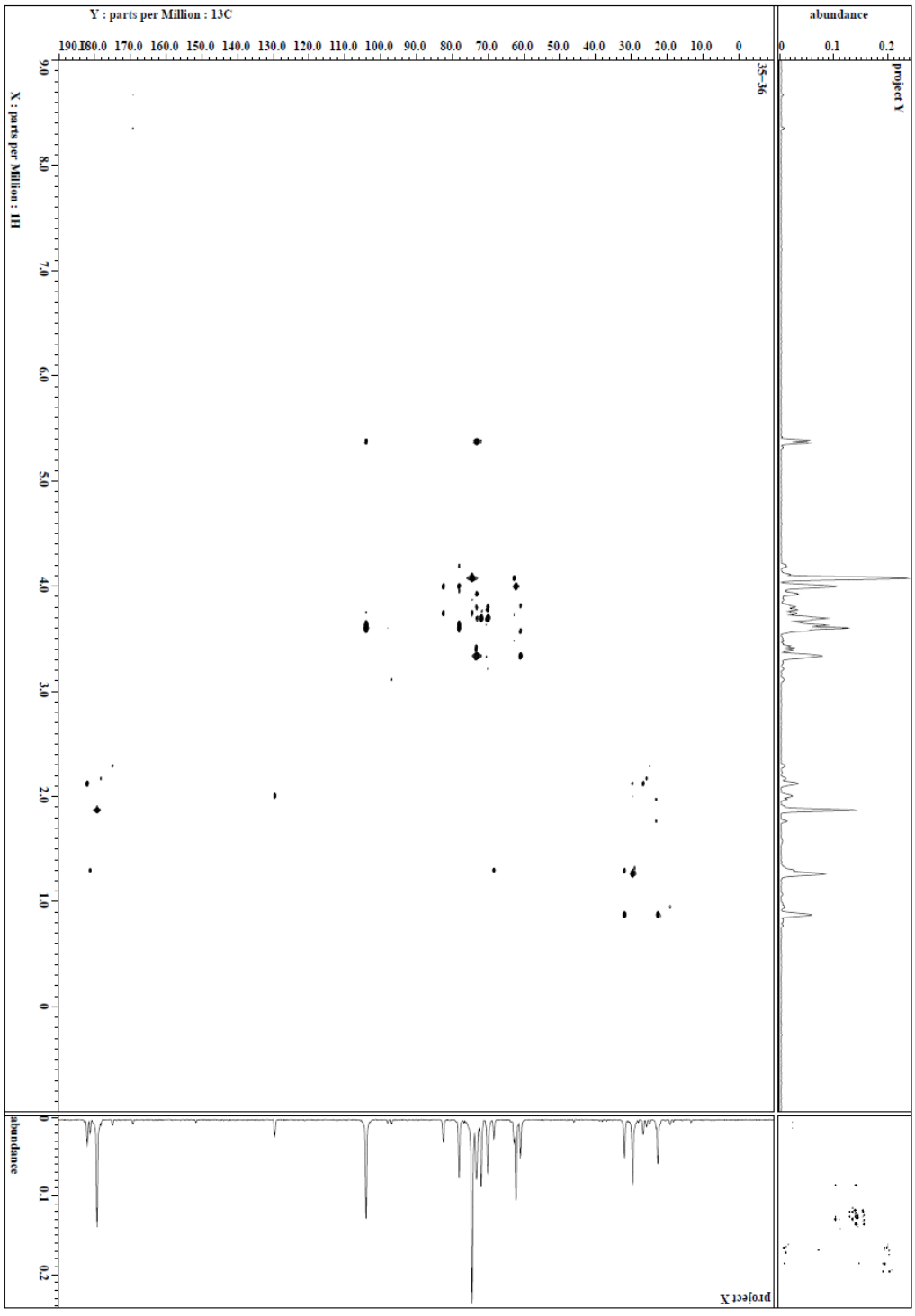


Figure 4.58 HMBC spectrum (600 MHz, CD_3OD) of 9.

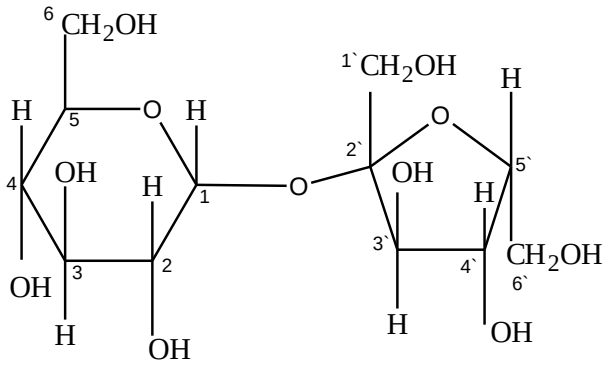


Figure 4.59 Proposed structure of 9.

4.2.4.2 Partial structure elucidation of compound 10

Compound **10** was isolated as a white solid substance from the crude glycosides extract of *T. davaeanum* TLC analysis of 10 on silica gel (2.5.5) showed no UV activity at 254 nm or 375 nm and a green colour spot (Rf = 0.25) after vanillin sulphuric acid spraying Figure 4.75 . Compound **10** has the molecular formula $C_{71}H_{111}O_{38}$ (m/z = 1572) as determined by an accurate mass measurement, the measured accurate mass was 1572.6754 and the calculated accurate mass was 1572.6793 Figiure 4.60. Figure 4.62 shows the ES- spectrum of **10.** ES+ mass spectrum of **10** is shown in Figure 4.63.

The ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data (600 MHz, CD_3OD) of **10** are shown in Table 4.15. The ^{13}CNMR spectrum of **10** (600MHz, CD_3OD) Figure 4.64 showed a signals for 68 carbons indicate the molecule has 68 carbon atoms. Among them seven correspond to methyl carbons at δ 13.4 C-24, 16.9 C-25, 17 C-26, 25.9 C-27, 15.9 C-28, 32.1 C-29, 23.7 C-30. The signals at δ 122.3 C-12 and 143 C-13 indicated a double bond between C-12 and C-13. Six signals in the downfield region at δ 105.1 C-1`, 99.9 C-1``, 100.2 C-1```, 111 C-1````, 102.2 C-1`````, 103.1 C-1`````` indicate the anomeric carbons. Furthermore, three carbonyl carbons were appeared further down field region at δ 171 C-31, 180 C-23 and 175.6 (the

position is not detected). Figure 4.65 is shown expansion of ^{13}CNMR spectrum of **10**.

The DEPT spectrum of **10** (600 MHz, CD_3OD) Figure 4.66 showed the existence of seven methyl groups (-CH_3) belonging to the aglycone part, C-24, C-25, C-26, C-27, C-28, C-29 and C-30 at δ values of 13.4, 16.9, 17, 25.9, 15.9, 32.1 and 32.7 respectively. The methylene groups (-CH_2-) belonging to the triterpene part were exhibited at δ 43 C-1, 20.1 C-6, 29 C-7, 23.3 C-11. Figure 4.67 is shown expansion of DEPT spectrum of **10**. The two methylene groups belonging to C-15 and C-16 are not seen in ^{13}CNMR spectrum, the reason for that could be because of overlapped ^{13}C NMR signals. It was reported before during the structure elucidation of novel triterpene glycosides isolated from *Muraltia heisteria* overlapped ^{1}HNMR signals of H-15 and H-16. In case of **10** overlapped signals occurre in both ^{1}HNMR and ^{13}CNMR spectra. Many signals were in agreement with a novel triterpene glycosides isolated from *Muraltia heisteria*.

The ^{1}HNMR spectrum of **10** (600 MHz, CD_3OD) Figure 4.68 showed that the aglycone part has an olean-12-ene skeleton from the signal at δ 5.33 (1H, t, H-12). In the high field region of the ^{1}HNMR spectrum of **10** signals of seven methyl groups appeared at δ 0.75 (3H, s, H-28), 0.85 (3H, s, H-29), 0.94 (3H, s, H-30), 1.22 (3H, d, *J* = 6.18, CH_3-Rha), 1.25 (3H, s, H-4), 1.28 (3H, s, H-25). Compound 10 was shown to contain 6 sugar moieties from the signals of the anomeric protons at δ 4.50 (1H, d, *J* = 7.73, H-1`), 5.02 (1H, d, *J* = unclear, H-1``), 5.25 (1H, d, *J* = H-1```), 5.10 (1H, d, *J* = 2.58, H-1````), 4.53 (1H, d, *J* = H- 1`````), and 4.42 (1H, d, *J* = 7.90, H-1``````). Figure 4.69 is shown expansion of ^{1}HNMR spectrum of **10**. Figure 4.70 is illustrated HMQC spectrum of **10**.

With regard to the structure elucidation of the aglycone part of **10** a significant HMBC correlations

Figure 4.71 gave evidence of a triterpene skeleton. The cross peak between the anomeric proton of the glucose moiety δ 4.50 (1H, d, *J* = 7.73, H-1`) to C-3 at δ 82 of triterpene part allowed the linkage in **10** between the aglycone part and the glucose moiety. The H-1 at δ 3.12 (2H, s, H-1) has three HMBC correlations, C-5 at δ 51., C-8 at δ 76 and C-9 at δ 67. H-2 at δ 4.01 (1H, m, H-2) is correlated to C-31 at δ 171. Also, the cross peak between H-3 at δ 3.54 (1H, s, H-3) and C-1 at δ 105.1 and C-2 at δ 71.1 of the glucose gave another evidence of the linkage between the aglycone part and glucose residue. Figure 4.72 is shown expansion of HMBC spectrum of **10**.

Other important HMBC correlations Figure 4.73 are between H-24 at δ 1.25 (3H, s, H-24), and C-10 δ 36.6, C-1 at δ 43, C-4 at δ 52, C-9 at δ 67, C-3 at δ 82, C-2 at δ 86 and C-23 at δ 180. Also H-25 at δ 1.28 (3H, s, H-25) is correlated to C-2 and C-3 at δ 86 and 180 respectively. In addition to the HMBC correlations between H- 29 at δ 0.85(3H, s, H-29) and C-20, C-22, C-21, C-19 at δ 30.1, 32.6, 35, 46.2 respectively. As well as the correlations between H-27 at δ 1.35 (1H, s, H-27) and C-25, C-27, C-18 and C-13 at δ 16.9, 25.9, 40.8 and 143 respectively. The other HMBC correlations are summarized in table 4.15.

The linkage between aglycone part and tetrasaccharide moiety was confirmed by reverse HMBC correlations between δ 4.50 (1H, d, H-1`) and δ at 82.1 C-3, and δ at 71.2 C-2`, this also permitted the position of C-2` after C-1` i.e. both carbons are in the same sugar unit. The anomeric proton at δ 5.02 (1H, d, H-1``) is correlated to C-2` δ 71.1 this permitted the linkage between first two sugar moieties.

Furthermore, the δ at 3.40 (1H, d, H-2``) is correlated to the second anomeric carbon at δ 99.9 C-1``. Therefore C-2`` δ 83 is the second carbon in this sugar unit. Because of third anomeric proton at δ 5.25 (1H,

d, H-1```) is correlated to C-2`` δ 83 this proved the linkage between third and second sugar units via (1→2) linkage.

The reverse HMBC correlations between the third anomeric proton at δ 5.25 (1H, d, H-1```) and fourth anomeric carbon at δ 111 C-1`````` and between fourth anomeric proton at δ 5.10 (1H, d, H`````)and third anomeric carbon C-1``` δ 100.2 allowed the linkage between sugar units 3 and 4. This linkage was further confirmed as the two anomeric carbons C-1``` δ 100.1 and C-1`````` δ 111 are correlated to the same carbon atom C-2``` δ 79.1.

The sugar units 5 and 6 are linked together and connected to the aglycone part at C-2 δ 86. this was confirmed by the HMBC correlation between anomeric proton at δ 4.53 (1H, d, H-1``````) and C-2 δ 86.

The proposed structure of **10** Figure 4.74 has molecular weight of 1538 and ES- mass spectrum of **10** figure suggest a molecular weight of 1574.8[M-1 = 1573.8].There is evidence of six sugars and agycone part could be triterpene. **10** has a comparable ^{1}H and ^{13}C NMR data with a mixture of trans and cis-trimethoxycinnamoyl triterpene glycosides isolated from Muraltia heisteria (Elbandy *et al*., 2002). Table 4.16 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) spectroscopic data comparison of 10 with ^{1}H NMR (600 MHz, C_5D_5N) and ^{13}C NMR (150 MHz, C_5D_5N) of a mixture of trans and cis-trimethoxycinnamoyl triterpene glycosides isolated from *Muraltia heisteria* (Elbandy *et al*., 2002). Further work needs to be carried out regarding to structure elucidation of **10**.

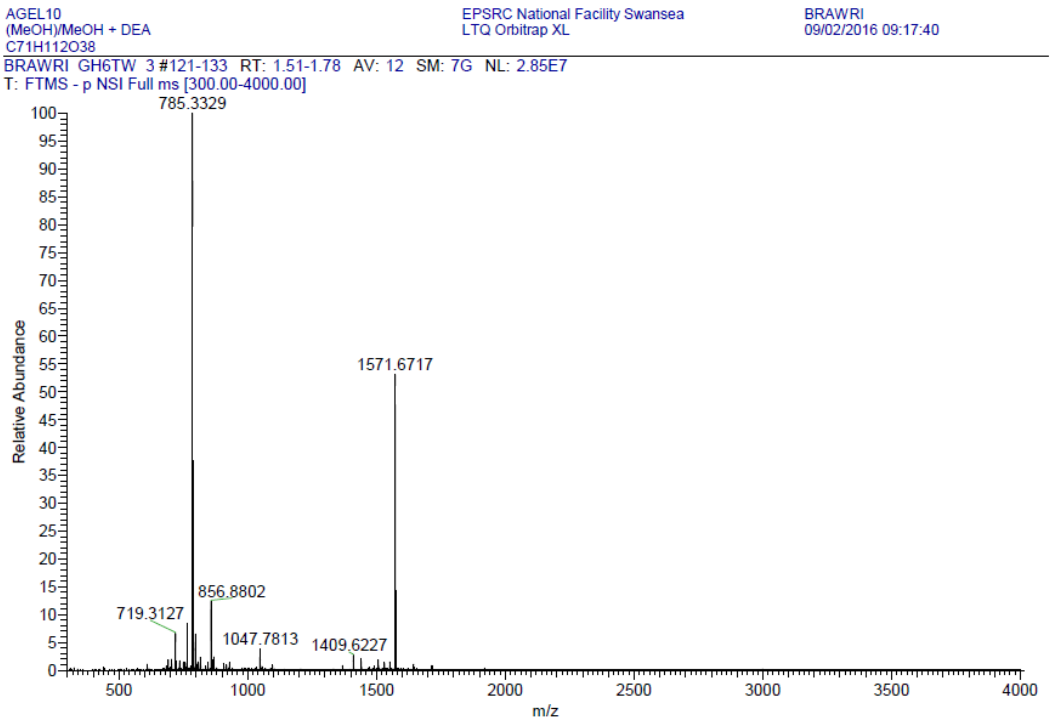


Figure 4.60 An accurate mass spectrum of 10.

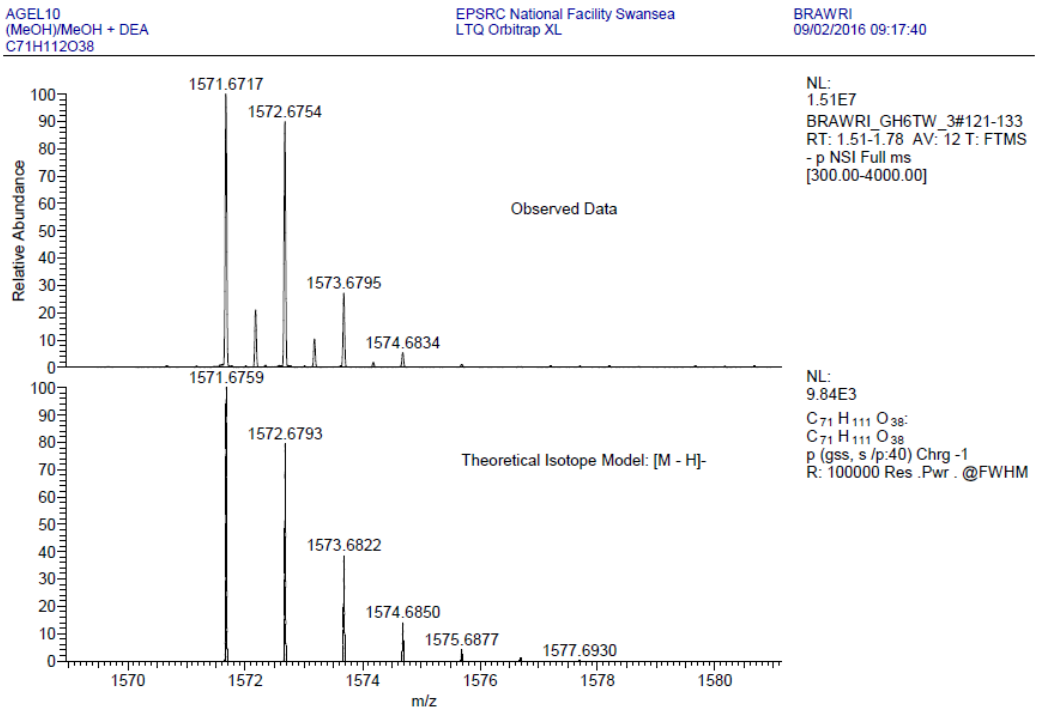


Figure 4.61 An expansion of accurate mass spectrum of 10.

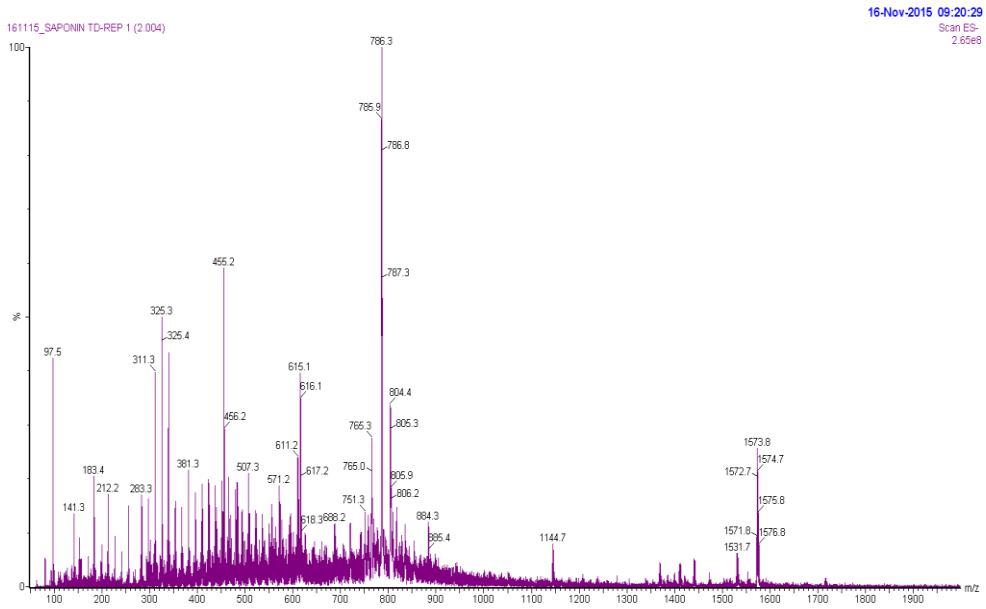


Figure 4.62 ES- mass spectrum of 10.

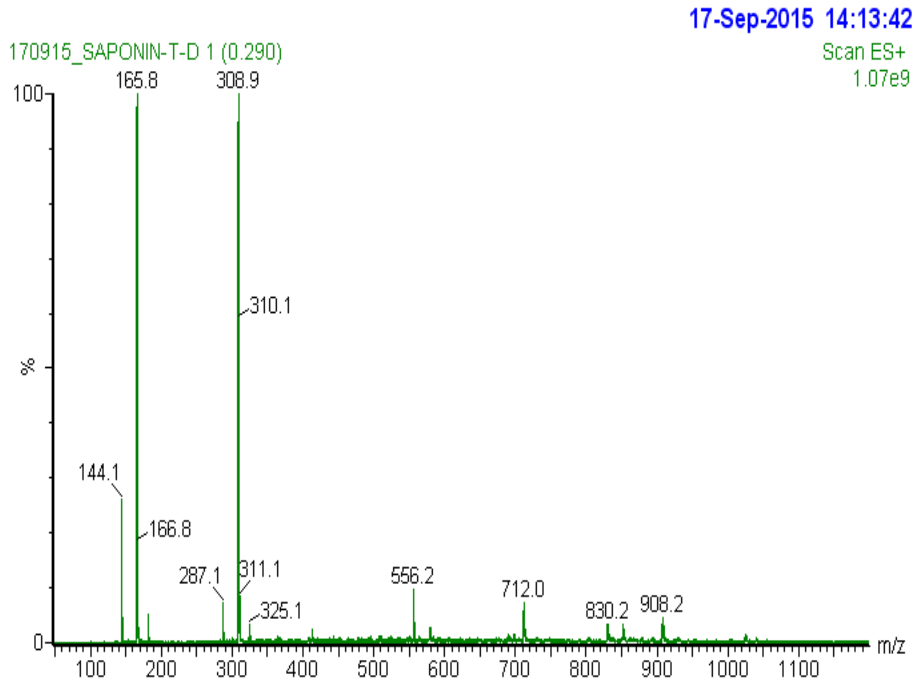


Figure 4.63 ES+ mass spectrum of 10.

Table 4.15 ^{1}HNMR, ^{13}CNMR, DEPT and HMBC data (600 MHz, CD_3OD) of 10.

No	^{1}H NMR (600 MHz, CD_3OD)	^{13}C NMR (600 MHz, CD_3OD)	DEPT (600 MHz, CD_3OD)	HMBC (600 MHz, CD_3OD)
1	3.12(2H, s, H-1)	43.3	CH_2	51.53, 67.31, 76.36
2	4.01	86	CH	170
3	3.54(overlapped)	82	CH	16.9, 71.2, 105
4	-	52	C	
5	3.30(overlapped)	51	CH	76, 44.81, 67.8, 105.12
6	?	20.1	CH_2	
7	4.02	29	CH_2	-
8	-	76	C	
9	3.88	67	CH	
10	-	36.6	C	
11	?	23.3	CH_2	
12	5.33(1H, t, H-12)	122.3	CH	
13	-	143	C	
14	-	48	C	
15		20.1	CH_2	
16		23.3	CH_2	
17		39.8	C	
18	3.05(1H, t, H-18)	40.8	CH	
19	3.27(2H, d, H-9)	46.2	CH_2	
20	-	30.1	C	
21	1.16(overlapped)	35	CH_2	15.98
22		32.6	CH_2	
23		180	C=O	
24	1.25(3H, s, H-24)	13.4	CH_3	36.22, 43.18, 47.59, 52.23, 67.7, 82.16, 86.3, 180.2
25	1.28(3H, s, H-25)	16.9	CH_3	180, 86.6
26	1.21(3H, s, H-26)	17	CH_3	-
27	1.35(3H, s, H-27)	25.9	CH_3	39.70, 40.79, 143.40
28	0.75(3H, s, H-28)	15.9	CH_3	32.51, 39.70, 41.79, 47.36
29	0.94(3H, s, H-29)	32.1	CH_3	30.19, 32.51, 35.30, 42.02, 46.89
30	0.85(3H, s, H-30)	23.7	CH_3	30.19, 35.53, 46.43
31	-	171	C=O	
32	3.78	59.9	CH_2	
33	3.88	61.2	CH_2	
34	3.84	62.7	CH_2	
35	3.57	64.2	CH_2	73.8, 76.5, 79.3
36	3.68	65.8	CH_2	
37	3.78	65.9	CH	
38	3.88	67.6	CH	
39	3.67	68.6	CH	72.8, 75.2, 103.2
40	3.48	68.8	CH	45
41	4.68	69	CH	
42	3.81	70.1	CH	105.1
43	3.81	70.1	CH	73.3, 82.1

Table 4.15 Continued

No	^{1}H NMR	^{13}C NMR	DEPT	HMBC
44	3.24	70.3	CH	77.0
45	3.95	70.7	CH	64.5, 111
46	-	71	CH	
47	3.32	72.7	CH	16.97, 68.93, 70.5, 73.8
48	3.21	72.9	CH	76.59, 103.27
48	3.93	73.0	CH	73.3, 93.0, 111.1
50	3.75	73.3	CH	
51	4.75	73.7	CH	73.3, 102, 170
52	4.45	73.9	CH	42.2
53	3.29	74.7	CH	83.0
54	3.43	75.1	CH	
55	-	76.5	CH	
56	3.95	76.6	CH	
57	-	76.9	CH	
58	-	78.2	C	
59	3.69	78.8	CH	
60	-	79.3	CH	
61	3.39	83	CH	
62	5.57	92.8	CH	
1`	4.50 (1H, d, *J* = 7.73 Hz)	105.1	CH	82.1
1``	5.02 (1H, d, *J* = unclear)	99.9	CH	67.54, 70.79, 74.04
1```	5.25 (1H, d)	100.2	CH	111.15, 68.4, 70.3, 79.1, 83
1````	5.10 (1H, d, *J* = 2.58 Hz)	111	CH	68.7, 73.8, 79.1, 100.44
1`````	4.53 (1H, d)	102.2	CH	86.10, 103.72
1``````	4.42(1H, d, *J* = 7.90 Hz)	103.1	CH	78.4

Table 4.16 ^{1}H and ^{13}C NMR (600 MHz, CD_3OD) data comparison of 10 with ^{1}HNMR(600 MHz, C_5D_5N) and ^{13}C NMR (150 MHz, C_5D_5N) of a mixture of trans and cis-trimethoxycinnamoyl triterpene glycosides isolated from *Muraltia heisteria* (Elbandy *et al*., 2002).

No	^{1}H NMR(600 MHz,CD_3OD) of 10	^{13}C NMR (600 MHz,CD_3OD) of 10	^{1}H NMR(600 MHz, C_5D_5N)	^{13}C NMR (150 MHz, C_5D_5N)
1	3.12(2H, s, H-1)	43.3	2.19, 1.28	43.9
2	4.01	86	4.57	70.0
3	3.54(overlapped)	82	4.50	86.6
4	-	52	-	53.1
5	3.30(overlapped)	51	2.21	52.3
6		20.1	1.85, 1.96	21.0
7	4.02	29	1.04, 1.28	33.6
8	-	76	-	40.7
9	3.88	67	2.27	49.0
10	-	36.6	-	36.5
11		23.3	Nd	23.8
12	5.33(1H, t, H-12)	122.3	5.83	127.8
13	-	143	-	139.0
14	-	48	-	47.8
15		20.1	Nd	24.2
16		23.3	Nd	24.0
17		39.8	-	46.8
18	3.05(1H, t, H-18)	40.8	3.14	41.8
19	3.27(2H, d, H-9)	46.2	1.27, 1.74	45.1
20	-	30.1	-	30.8
21	1.16(overlapped)	35	1.85, 2.3	34.0
22		32.6	1.73, 1.85	32.0
23		180	-	180.8
24	1.25(3H, s, H-24)	13.4	1.86(s)	14.4
25	1.28(3H, s, H-25)	16.9	1.47(s)	17.2
26	1.21(3H, s, H-26)	17	1.08(s)	18.7
27	1.35(3H, s, H-27)	25.9	2.85, 4.12	64.2
28	0.75(3H, s, H-28)	15.9	-	176.3
29	0.94(3H, s, H-29)	32.1	0.77(s)	32.7
30	0.85(3H, s, H-30)	23.7	0.78(s)	23.4
31	-	171		
32	3.78	59.9		
33	3.88	61.2		
34	3.84	62.7		
35	3.57	64.2		
36	3.68	65.8		
37	3.78	65.9		
38	3.88	67.6		
39	3.67	68.6		
40	3.48	68.8		
41	4.68	69		
42	3.81	70.1		

Table 4.16 Continued

No	^{1}H NMR	^{13}C NMR	DEPT	HMBC
43	3.81	70.1	CH	73.3, 82.1
44	3.24			
45	3.95			
46	-			
47	3.32			
48	3.21			
49	3.93			
50	3.75			
51	4.75			
52	4.45			
53	3.29			
54	3.43			
55	-			
56	3.95			
57	-			
58	-			
59	3.69			
60	-			
61	3.39			
62	5.57			
1`	4.50 (1H, d, *J* = 7.73 Hz)			
1``	5.02 (1H, d)			
1```	5.25 (1H, d, J Hz)			
1````	5.10 (1H, d, *J* = 2.58 Hz)			
1`````	4.53 (1H, d, J = Hz)			
1``````	4.42(1H, d, *J* = 7.90 Hz)			

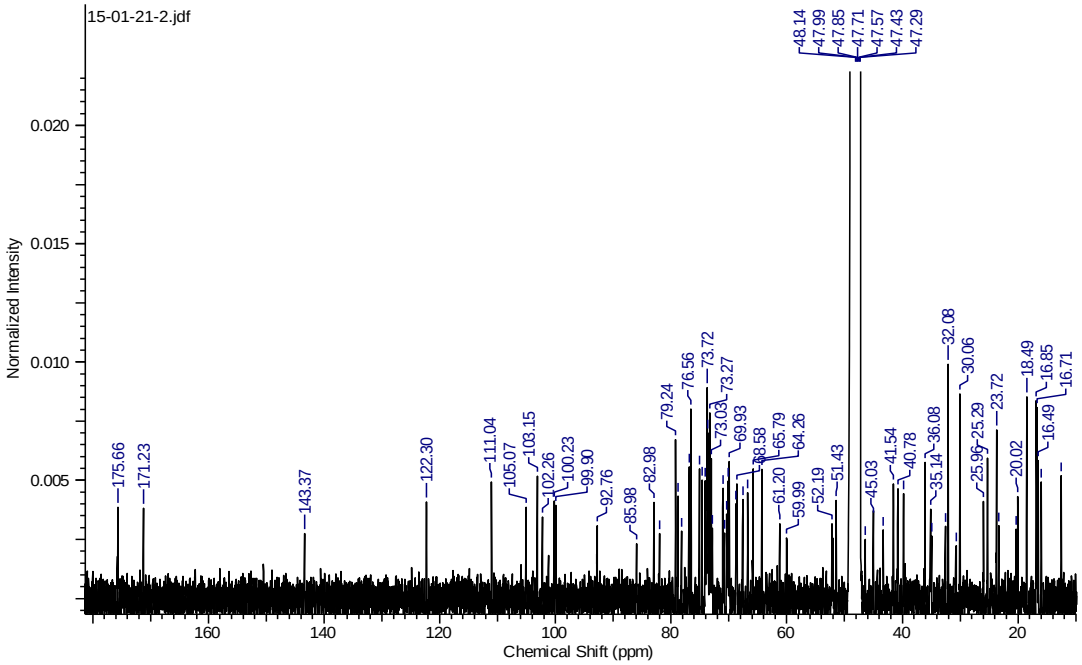


Figure 4.64 ^{13}C NMR spectrum (600 MHz, CD_3OD) of 10.

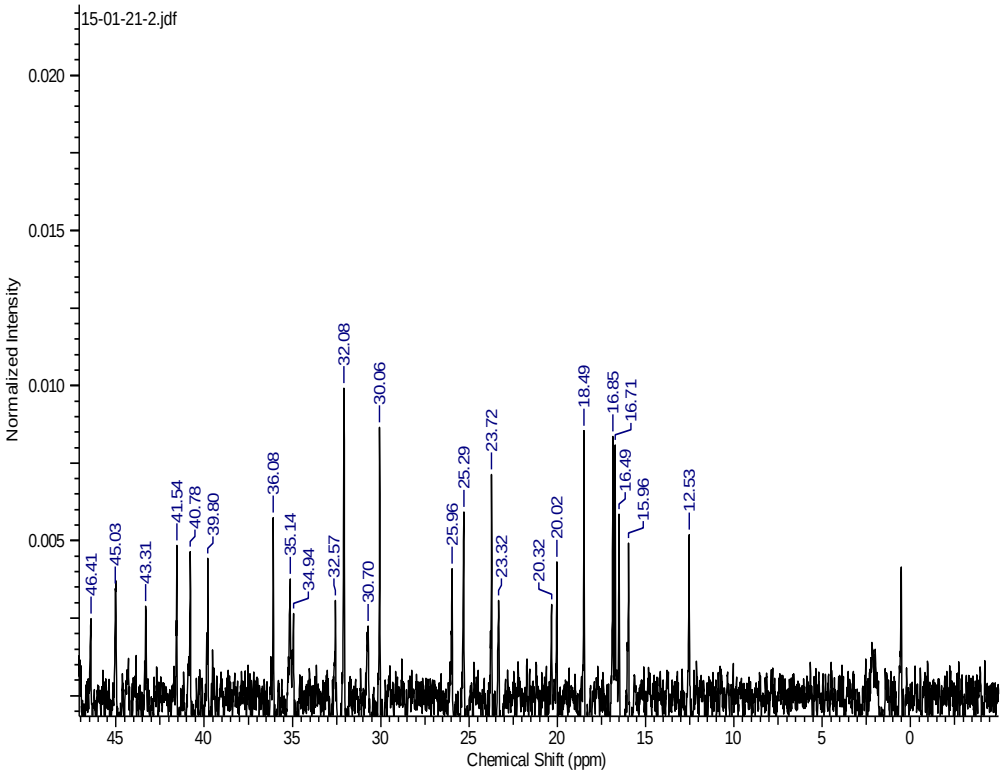


Figure 4.65 Expansion of ^{13}C NMR spectrum (600 MHz, CD_3OD) of 10.

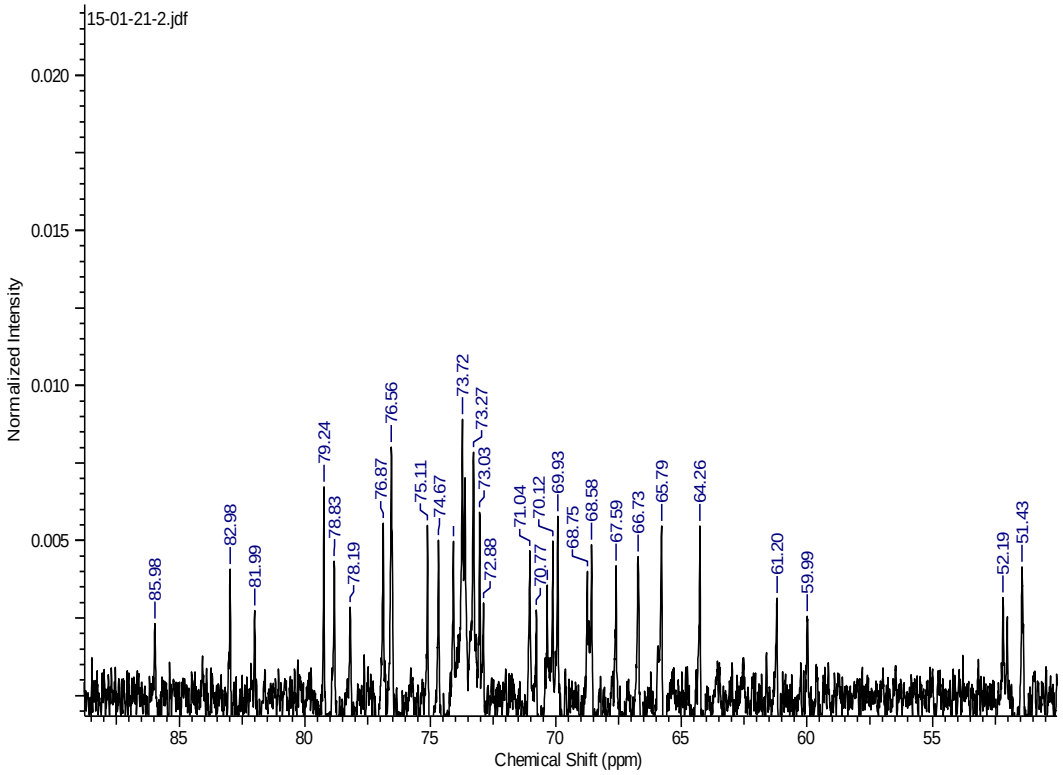


Figure 4.62 Continued.

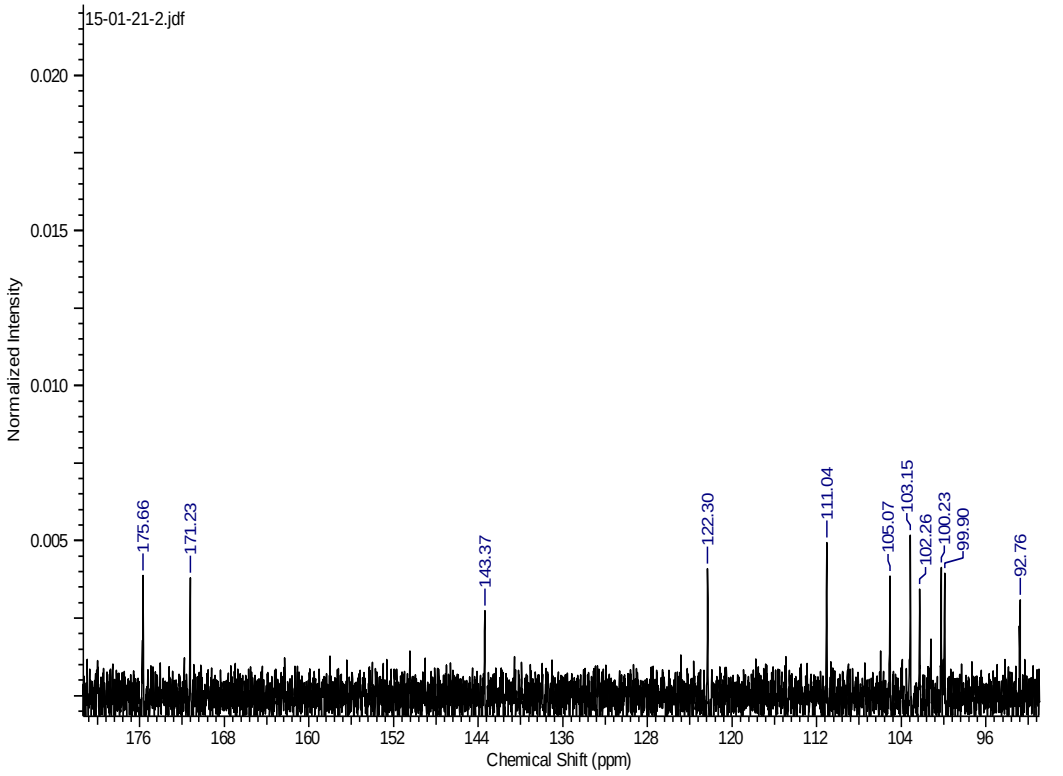


Figure 4.65 Continued.

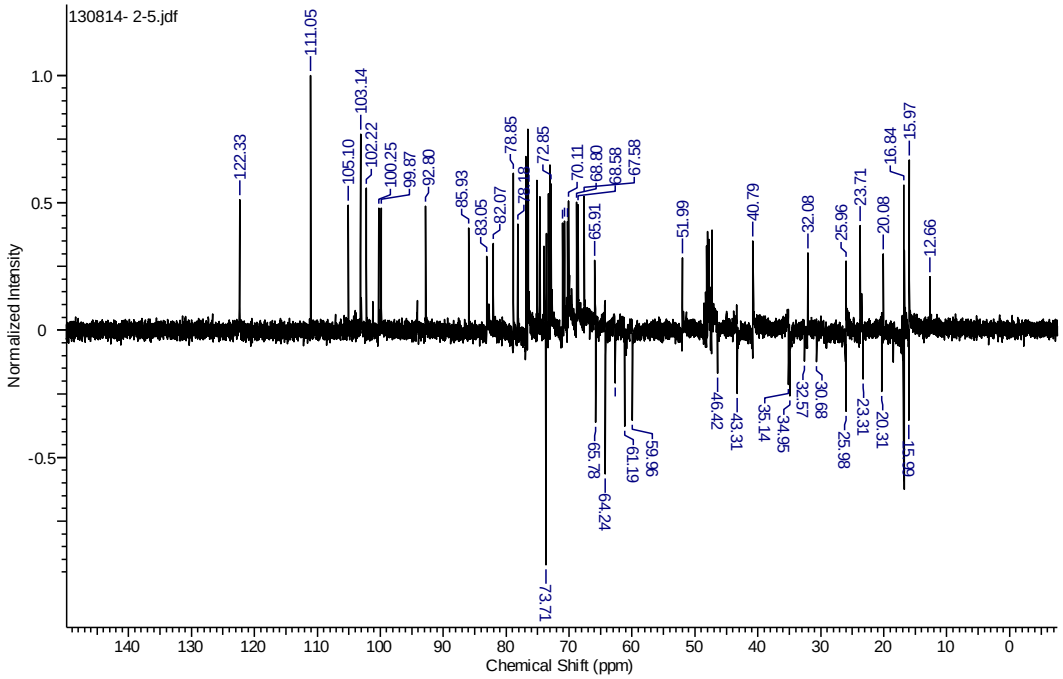


Figure 4.66 DEPT spectrum (600 MHz, CD_3OD) of 10.

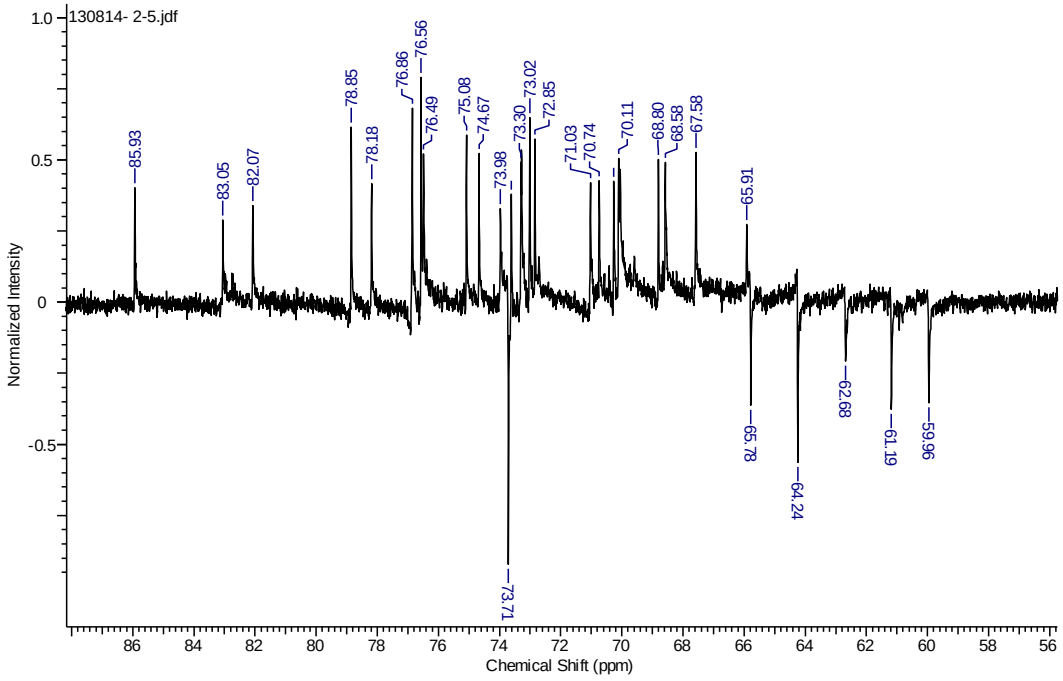


Figure 4.67 Expansion of DEPT spectrum (600 MHz, CD_3OD) of 10.

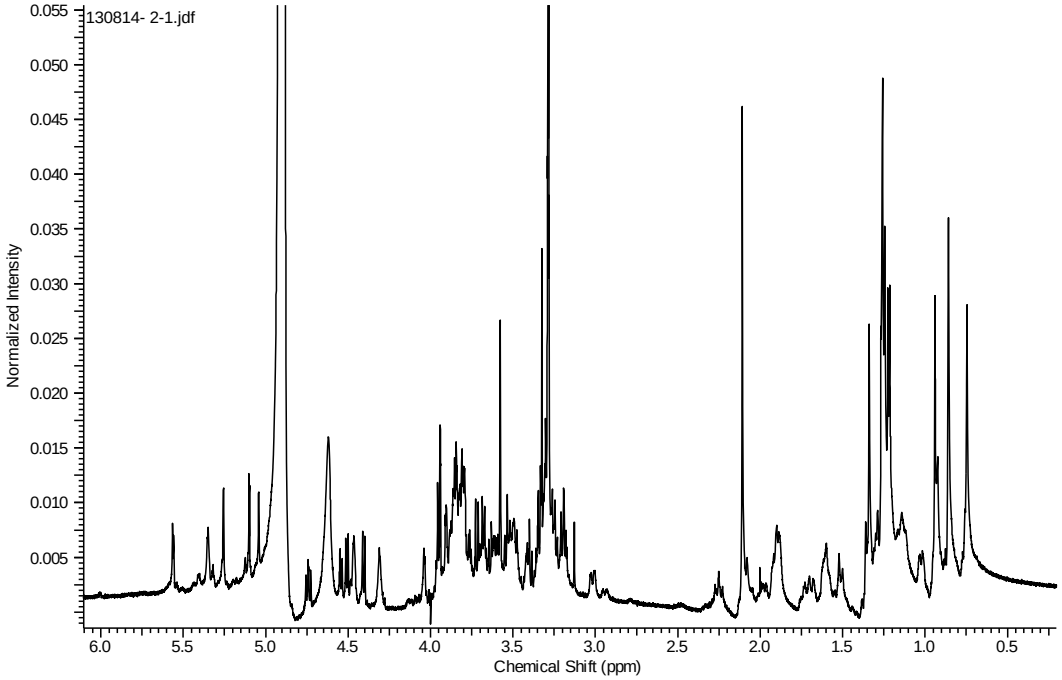


Figure 4.68 ^{1}H NMR spectrum 0 (600 MHz, CD_3OD) of 10.

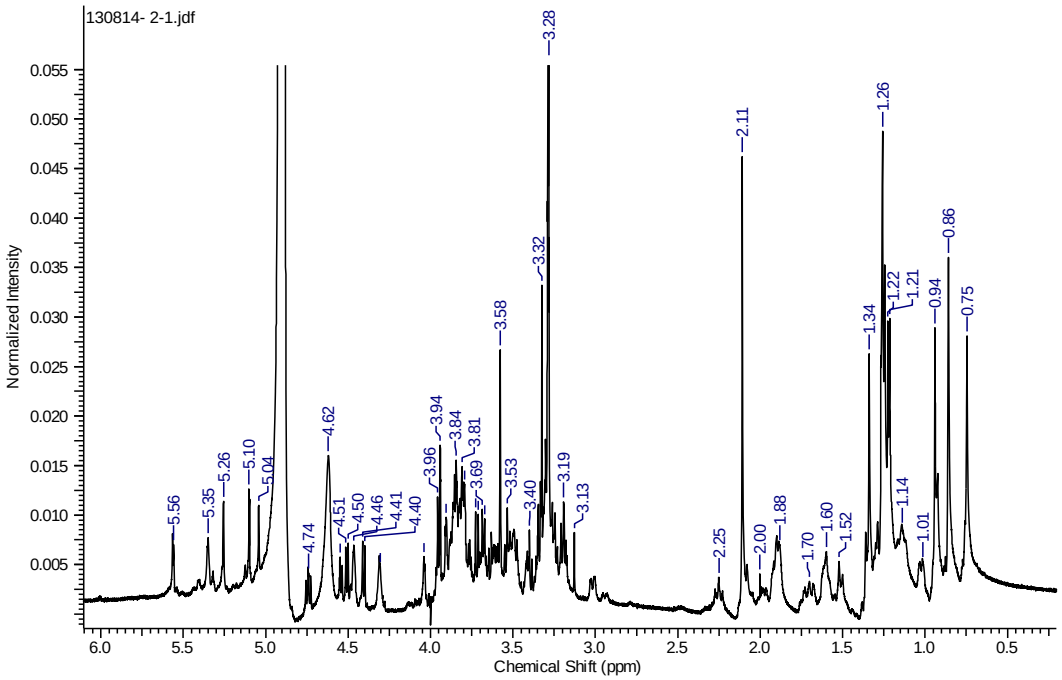


Figure 4.69 Expansion of ^{1}H NMR spectrum (600 MHz, CD_3OD) of 10.

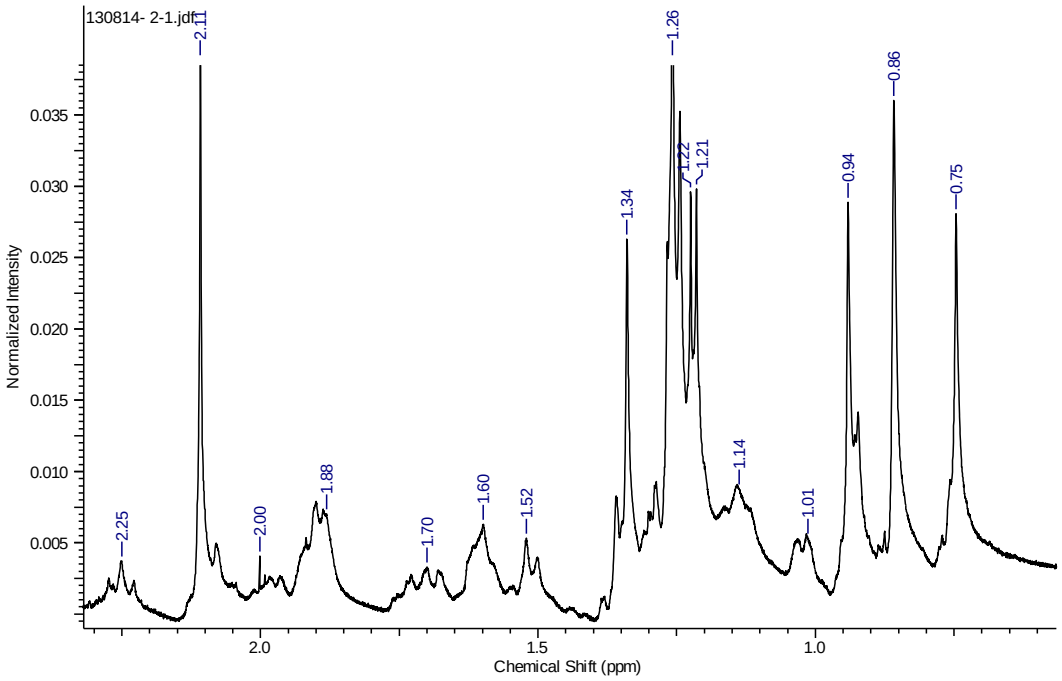


Figure 4.69 Continued.

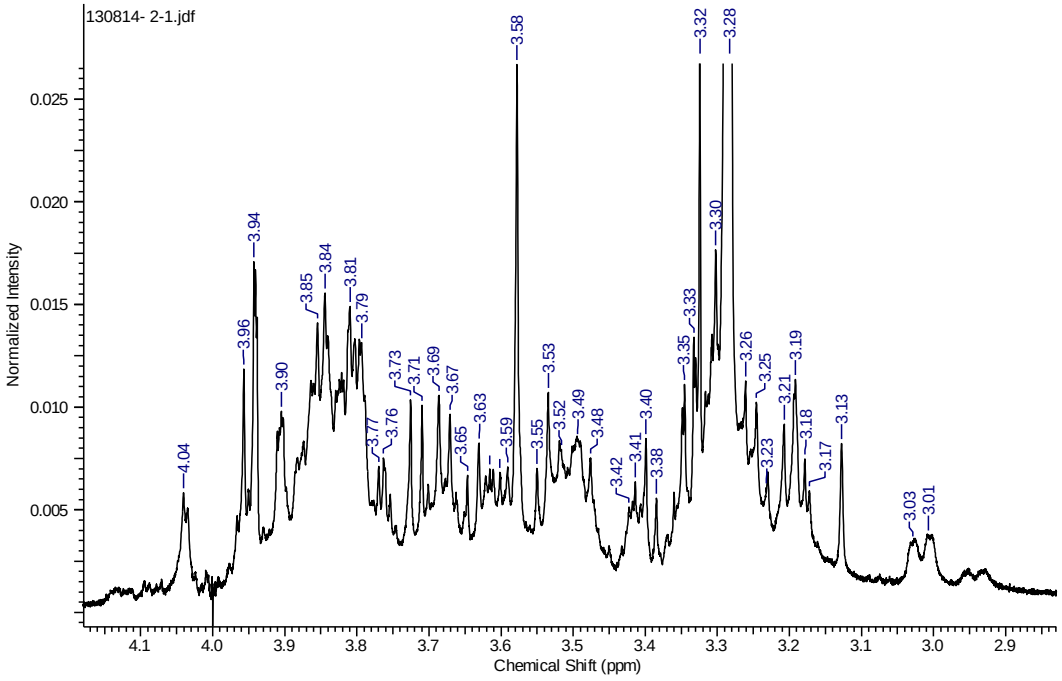


Figure 4.69 Continued.

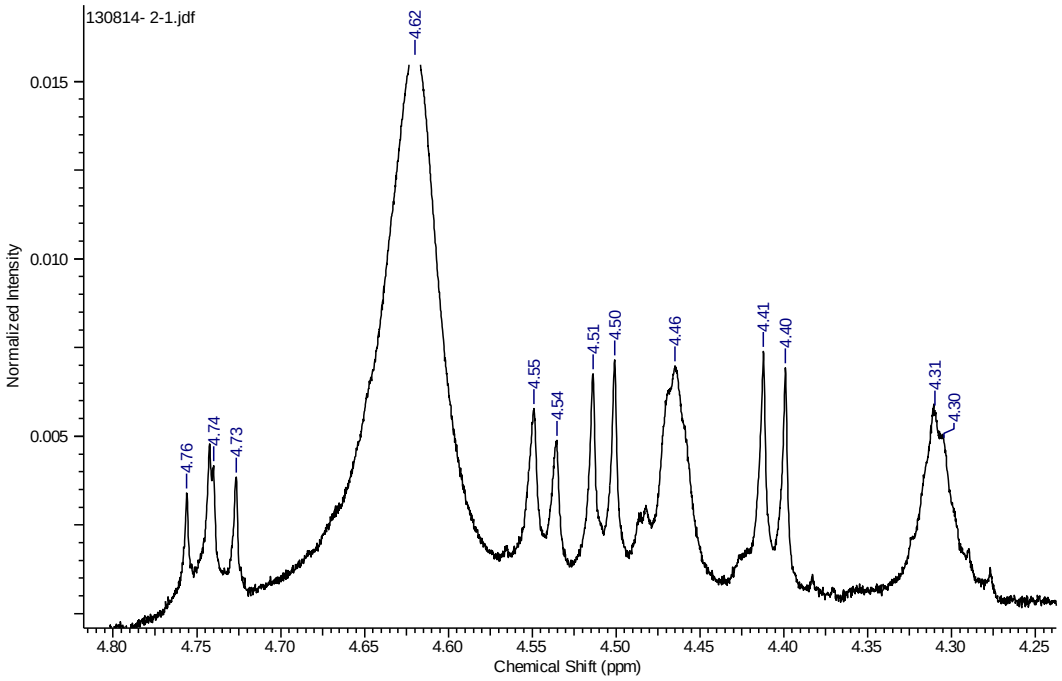


Figure 4.69 Continued.

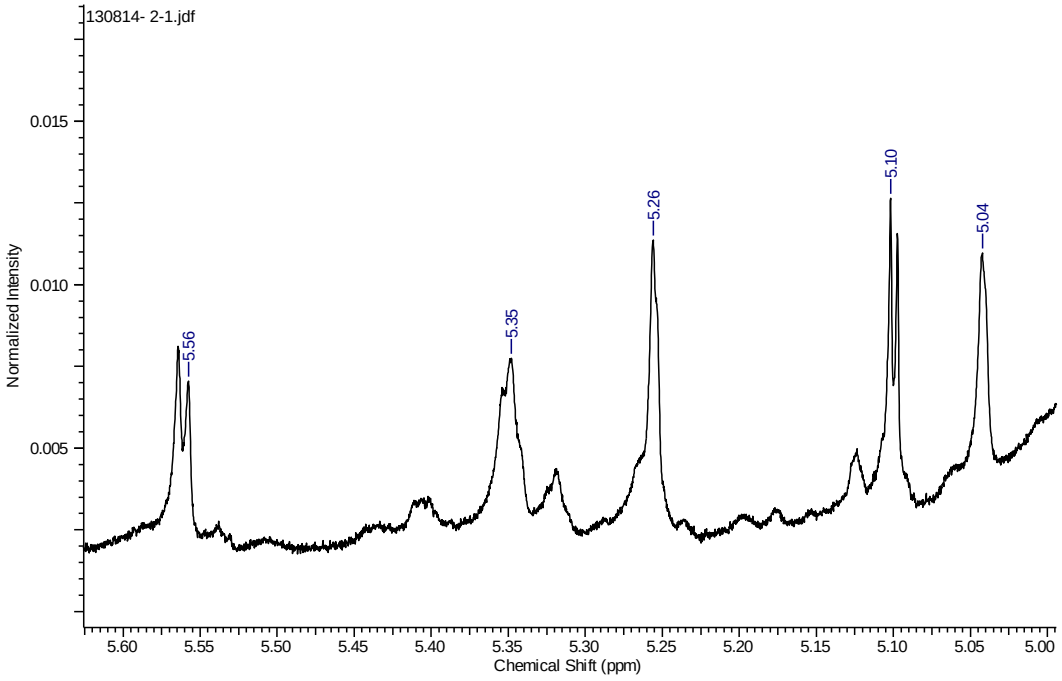


Figure 4.69 Continued.

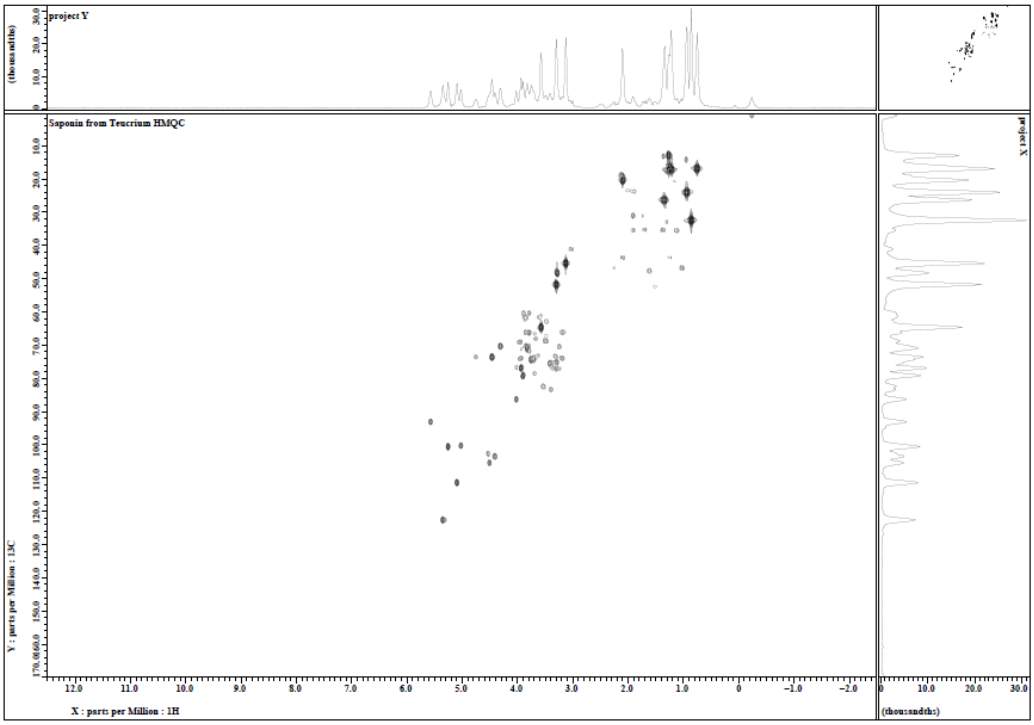


Figure 4.70 HMQC spectrum (600 MHz, CD_3OD) of 10.

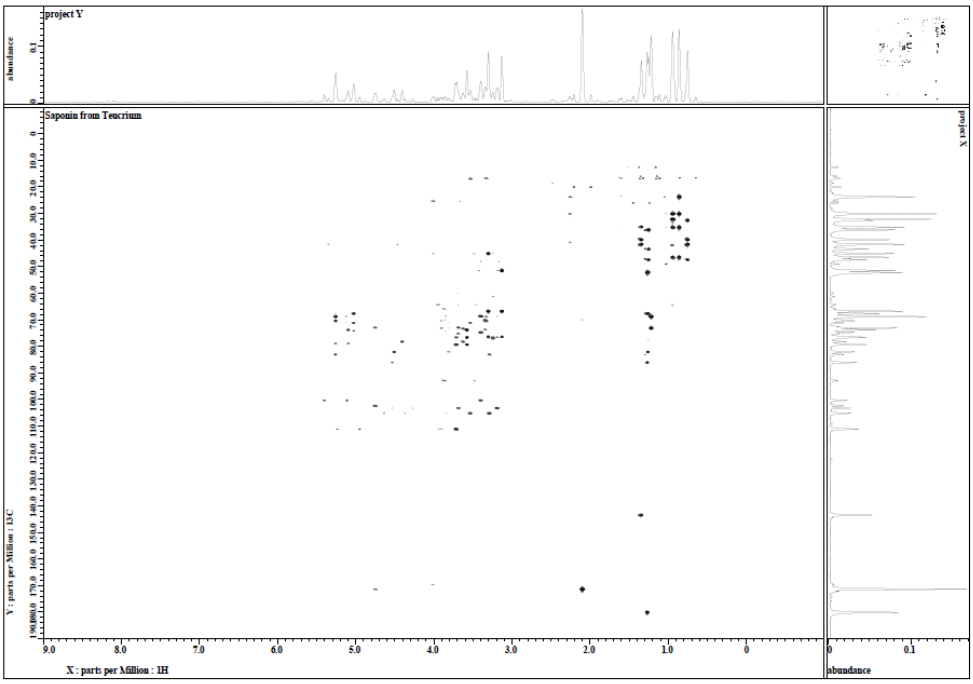


Figure 4.71 HMBC spectrum (600 MHz, CD_3OD) of 10.

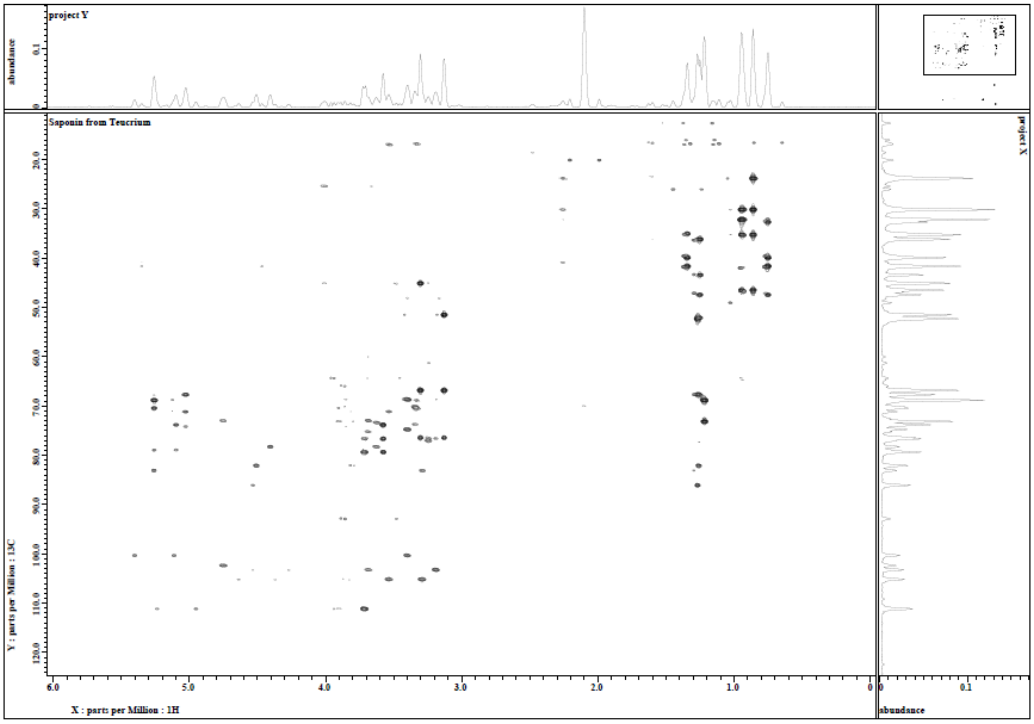


Figure 4.72 Expansion of HMBC spectrum (600 MHz, CD_3OD) of 10.

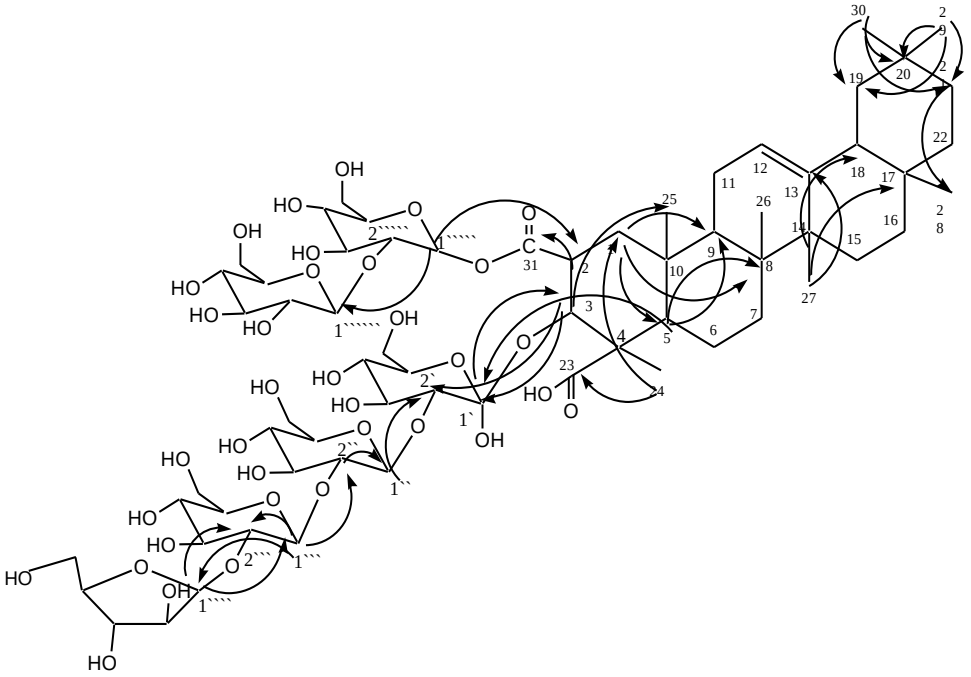


Figure 4.73 HMBC correlations of 10.

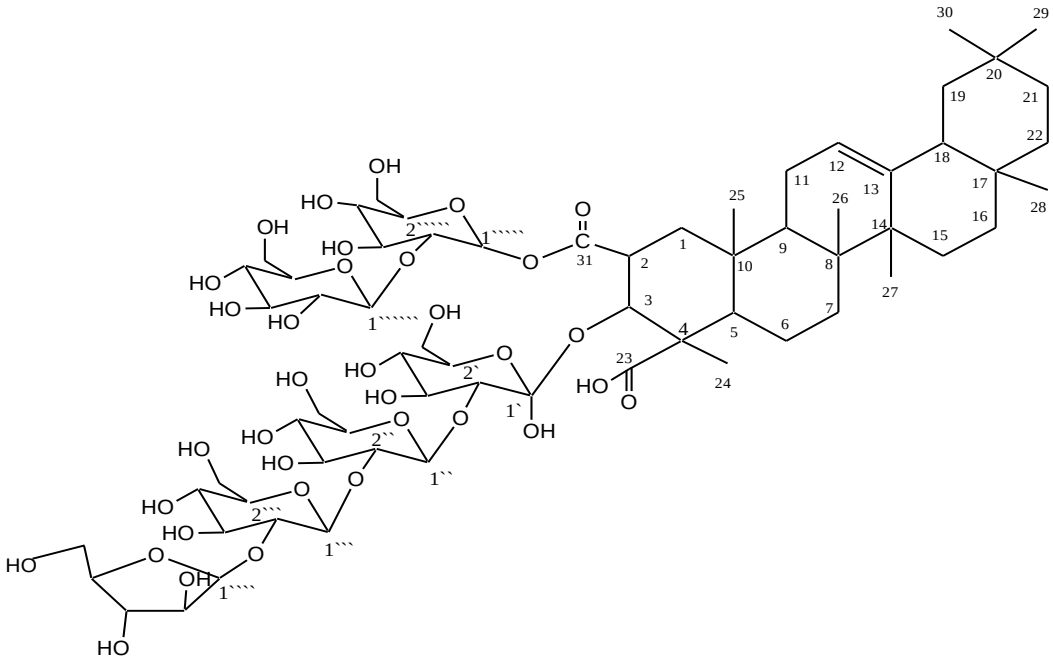


Figure 4.74 Suggested structure of compound 10.

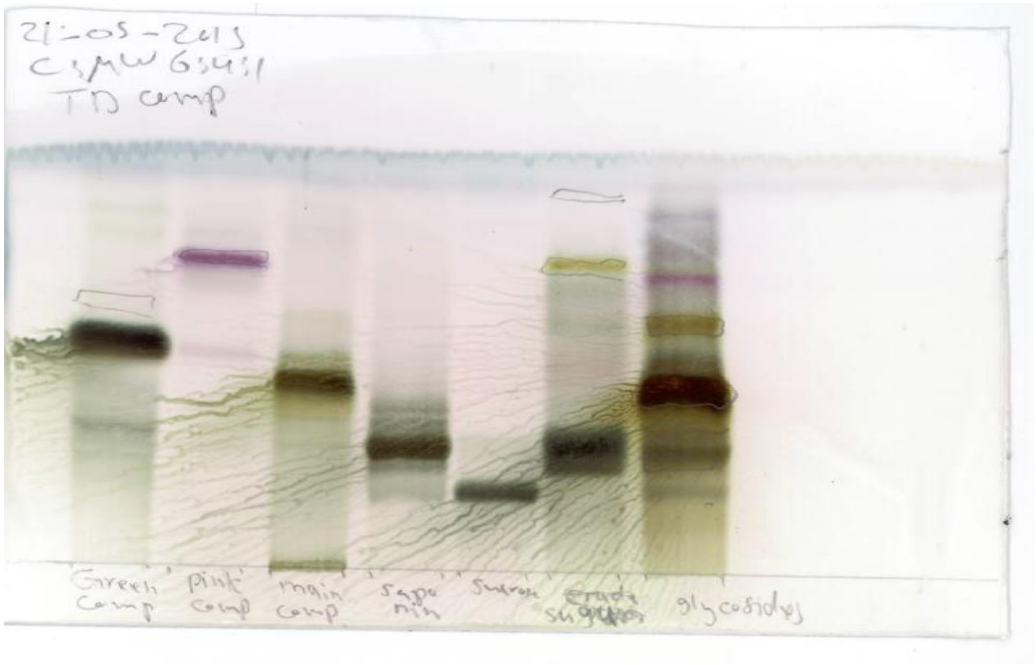


Figure 4.75 Glycosides isolated from the crude glycosides of *T. davaeanum* (The bands from left to right are compounds 7, 8, 6, 10 and 9. The last two bands are crude glycosides extract).

5 Assessment of total polyphenol contents, antioxidant and cytotoxic activities of studied plants extract and/or isolated compounds.

5.1 Quantification the total phenol content

5.1.1 Introduction

Polyphenols comprise a large class of phytochemicals that have a variety of important biological activities. Polyphenols consist of one or more aromatic rings attached to one or more hydroxyl groups. In many cases one or more sugar moieties are commonly attached (Miniati, 2007, Dai and Mumper, 2010). Polyphenols are broadly distributed in plant kingdom and more than 8000 phenolic compounds have been discovered (Tsao, 2010).

Phenolic compounds are distributed among plant foods such as fruits, vegetables, olive, chocolate, legumes, cereals (Dai and Mumper, 2010), edible and inedible plants (Wojdyło et al., 2007), derived foods, e.g. juices, oils and wines (Miniati, 2007). They are secondary metabolites of plants (Dai and Mumper, 2010).

An imbalance in free radicals production may lead to cells damage that causes deleterious changes in blood vessels, inflammatory conditions, neoplastic diseases, cataract formation and neurodegeneration. Their presence in food constituents could reduce the oxidation process and prevent free radical activity. The results showed that these diseases can be prevented by consuming a sufficient quantity of food which containing natural antioxidant such as polyphenols, which are considered to be strong antioxidant than antioxidant vitamins. The main properties of polyphenols (especially those with hydroxyl group in position ortho or

para) is the compounds enter easily into redox reactions. (Cieślik et al., 2006).

It has been reported that the total weight of polyphenols which should be consumed daily is 1-2 g. (In Poland they considered the weight of polyphenols to be consumed daily to 0.032g, while in Finland, Japan and USA they are 0.003 g, 0.068 g and 1.1 g respectively) (Cieślik et al., 2006).

Polyphenols have been reported to have antioxidant activity and many uses such as nutrition, beverages, repellents, cosmetics, medicine, flavouring, dyeing, fragrances (Miniati, 2007), antimicrobial, antimutagenic effects, anti-inflammatory, hypolipidemic (Wojdyło et al., 2007). Their presence in plants acts to protect them from ultraviolet radiation or aggression by pathogens, predators and parasites in addition to contributing to plant colours (Dai and Mumper, 2010). The natural flavonoid (-)-epigallocatechin gallate (EGCG), the major constituent of green tea was found to be responsible for the potential green tea in prevent of lung cancer (Khan and Mukhtar, 2015).

On reviewing the literature on the phytochemistry of *Sanicula europaea* and *Teucrium* genus it is found that the species are rich in phenolic compounds that are known for their health benefits and antioxidant activity. Therefore the aim of this study was to determine the total phenol content of 50% ethanol extract of the aerial parts of both species using Folin-Ciocalteu's method.

5.1.2 Materials

Agilent 8543 UV/Vis spectrometer and Biochem Anal Software for Agilent Chem Station or equivalent were from Sigma, UV/Vis spectroscopy cells or equivalent (Multi-Wrist Shaker, Model 3589) obtained from Sigma, balance accurate to 0.0001 g, volumetric flasks (size 25ml, 50ml, 100ml)

were from Life Technologies, Reusable class B volumetric pipets were from Life Technologies. Gallic acid standard, Folin Ciocalteu's phenol reagent, sodium carbonate HPLC grade, water HPLC grade, ethanol HPLC grade all were from Sigma.

5.1.3 Extraction method

The determination of total polyphenol content was carried out according to Folin Ciocalteu's method (Waterhouse, 2001). The ethanol extract of *S. europaea* and *T. davaeanum* was prepared by extracting 15 g of dried aerial parts plant materials with 150 ml of 50 % ethanol using sonicator for 20 minutes then extracted again using a centrifuge for 10 minutes. The liquid layer was carefully removed, filtered and air removed with N_2 gas (to prevent the oxidation of polyphenols) for determination of total polyphenols content and antioxidant activity.

5.1.4 Procedure of experimental work

To determine the total polyphenol content of *Sanicula europaea* and *Teucrium davaeanum* via Folin-Ciocalteu's method a standard curve of gallic acid was first plotted by measuring the absorbance at several concentration of gallic acid. The bsorbance of several concentration of 50% ethanol extract of *Sanicula europaea* and *Teucrium davaeanum* were measured on the same day.

Gallic acid concentrations of (0, 0.05, 0.15, 0.25 and 0.5) mg/ml were prepared by pipetting (0, 1, 2, 3 and 5) ml of gallic acid solution (preparation of reagents section) into 100 ml volumetric flasks, separately then diluted to volume with distilled water. The volumetric flask which has 0 ml of diluted gallic acid was the blank solution.

From the first concentration of gallic acid (0 mg/ml) 20 µl was pipetted into UV cuvette and 1.58 ml water and 50 µl of Folin-Ciocalteu's phenol reagent was added, the cuvette contents then mixed well. After 1 minute 300 µl of sodium carbonate (20 %) was added. The cuvette contents were mixed again, covered with Aluminium foil then left for two hours, this solution is called the blank solution. This was repeated for the other concentrations of Gallic acid. The absorbance of gallic acid at 734 nm is an average of three determinations (0.05, 0.015, 0.25 and 0.5) mg/ml.

An ethanol extract of *S. europaea* was prepared by extract 15 g of dried plant material with 150 ml of 50 % ethanol using sonicate for 20 minutes then extracted again using centrifuge instrument for 10 minutes the liquid layer was carefully removed and covered with N_2 gas to prevent the oxidation of polyphenols.

Several volumes were measured out from the extract (0, 1, 2, 3, 5 and 10) ml into 10 ml volumetric flasks, and then diluted to volume with distilled water. From each solution 20µl was pipette into separate cuvettes and to each 1.58 ml water, 100 µl Folin reagent mixed well, after 8 minutes 300 µl of 20% sodium carbonate solution was added. After 2 hours the absorbance was measured at 760 nm.

This process was repeated for two samples of *T. davaeanum,* 15 g of leaves and flower and 15 g of stem.

5.1.5 Results and discussion

The gallic acid standard curve is shown in Figure (5.8). The standard curve will be used to determine the total phenol content of *Sanicula europaea* and *Teucrium davaeanum* according to gallic acid equivalents.

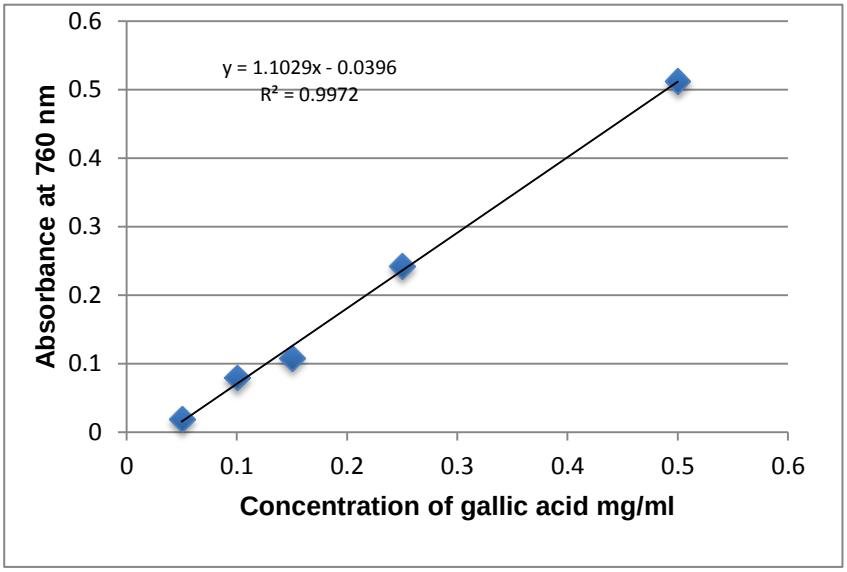


Figure 5.1 Gallic acid standard curve.

Absorbance of 10 mg/ml of *S. europaea* dried plant material (0.507) fits in the absorbance range of the standard curve (SC), therefore this value can be used to calculate the concentration of extract. Therefore, SC equation was applied to calculate the percentage of polyphenol in the ethanol extract of *S. europaea.* Figure 5.9 is shown the curve obtained from absorbance of several concentrations of *S. europaea.*

X=(y+0.0396)/1.1029,

Where X = conc. of polyphenols in *S. europaea* in 10 mg/ml.

Y = the absorbance of *S. europaea* extract

X = (0.507 + 0.0396)/1.1029.

X = 0.496 mg/ml

Therefore, the total polyphenol content of *S. europaea* is 49.6 mg/100 ml. Factor in for weight of solid per volume of solvent; ethanol extract was prepared as 15g of material in 150 ml of 80 % methanol. It had a concentration of 100 mg/ml. hence % of polyphenols per mg of physical material = (0.496/10) x 100 = 4.956 %.

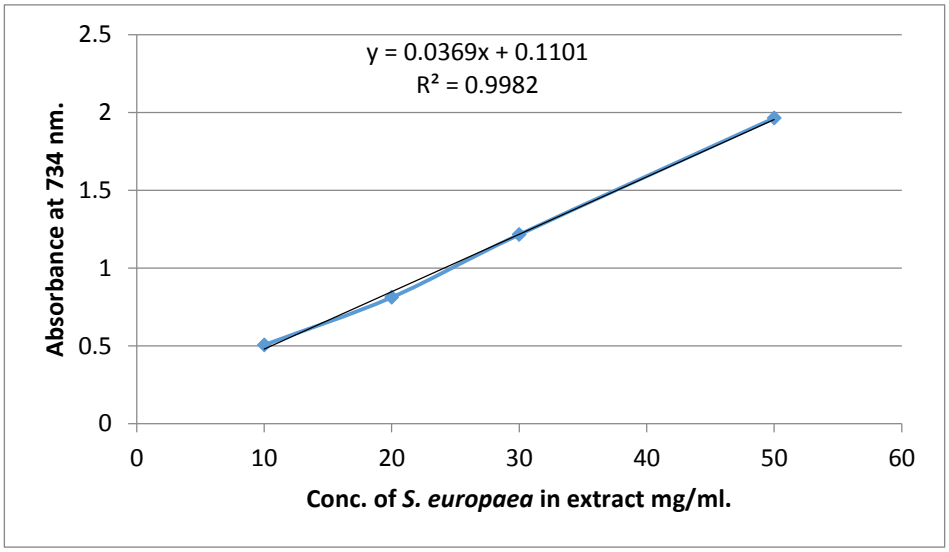


Figure 5.2 Conc. vs absorbance of *S. europaea* extract.

Calculation of total phenols content of *T. davaeanum* 50 % ethanol extract by measure the absorbance of several conc. of ethanol extract at 734, and draw a curve. Figure 5.10 conc. of *T. davaeanum* leaves and flowers extract vs absorbance. The absorbance of 1 ml (0.235) and 2 ml (0.484) values are in the absorbance range of the standard curve, therefore these values can be used to calculate the concentration of extract. Rearrange SC equation.

X = (y + 0.0396) / 1.1029

X = (0.235 + 0.0396) / 1.1029 =0. 24898 in 10 mg/ml

X = (0.484 + 0.0396) / 1.1029 = 0.47505 in 20 mg/ml

The percentage of total polyphenols was calculated as the average of the two values (0.24898 + 0.47505) / 2 = 0.362 this extract was prepared as 15 g of material in 150 ml of 50 % ethanol. It had a concentration of 100 mg/ml. hence polyphenol per mg of physical material = (0.362/30) = 0.012 mg/ml, thus 1.20 %.

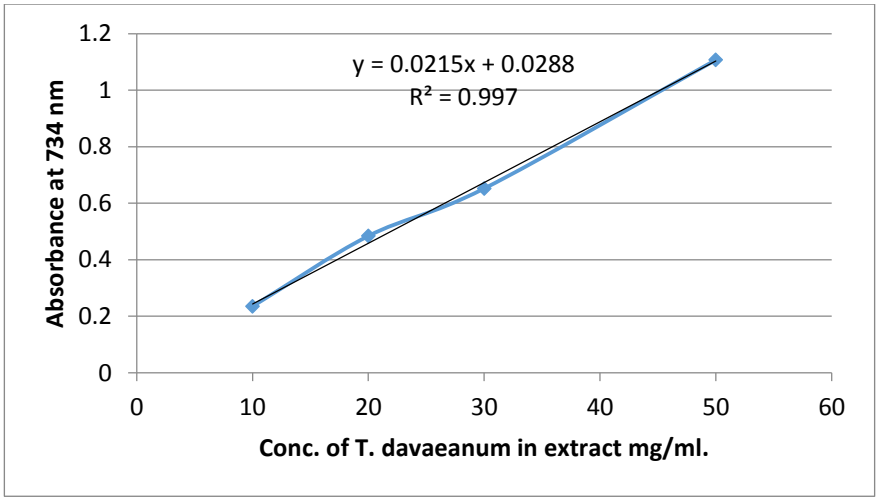


Figure 5.3 Conc. of *T. davaeanum* leaves and flowers vs absorbance.

Calculation of total phenols content of *T.* davaeanum stem ethanol extract by plot experimental data and check the linearity. Figure 5.4 is shown conc. of T. davaeanum stem extract vs absorbance. The absorbance of 2 ml (0.244) and 3 ml (0.394) fill in the absorbance range of the standard curve, therefore these values can be used to calculate the concentration of extract.

X=(y+0.0396)/1.1029.

X = (0.243 + 0.0396) / 1.1029 = 0.2562 of 20 mg/ml

X = (0.394 + 0.0396) / 1.1029 = 0.3931 of 30 mg/ml

The percentage of total polyphenols was calculated as the average of the two values (0.2562 + 0.3931) / 2 =0.3246 in 50 mg (20+30) dried plant material. Factor in for weight of solid per volume of solvent in the extract, this extract was prepared as 15 g of material in 150 ml of 50 % ethanol. It had a concentration of 100 mg/ml. hence polyphenol per mg of physical material (0.325/50) = 0.0065 mg, thus 0.65 %.

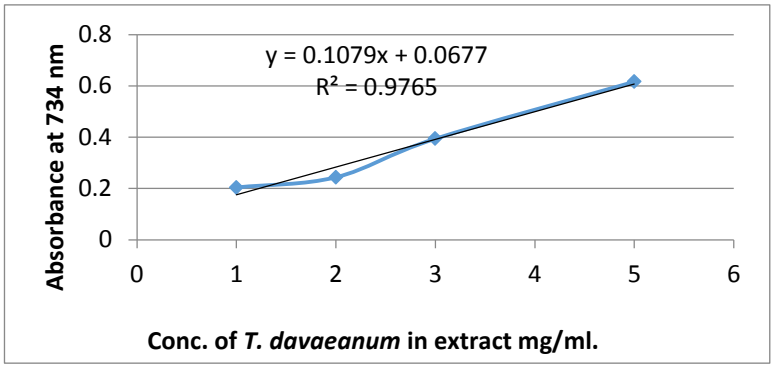
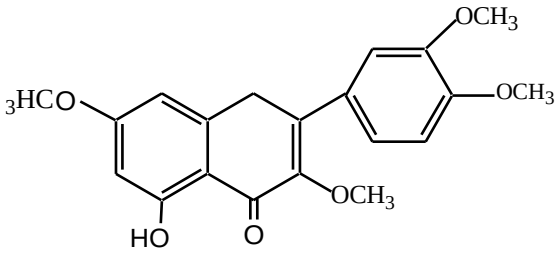
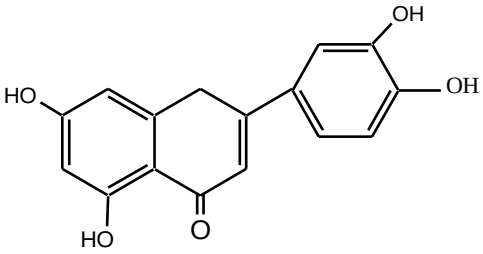


Figure 5.4 Conc. of *T. davaeanum* stem extract vs absorbance.

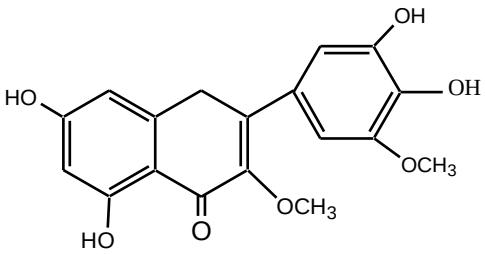
On reviewing the literature on *Sanicula europaea* two known phenolic acids, rosmarinic acid, and caffeic acid were isolated by Arda *et al*. (1997) as a major compounds (Figure 1.9). Rosmarinic acid was also isolated again during this study (section 3.4.6). These constituents can be counted as a part of the total phenol contents. Five known flavonoids, 3,7,3`,4` tetra-methoxy, 5-hydroxy flavone, 5,7,3`,4` tetra-hydroxy flavone (luteolin), 3.5` dimethyl myricetin, 5-hydroxy, 3`,4`,6,7-tatra-methoxy flavone and Luteolin-7-O-gluco-glucosyl-3`-o-rhamnoside Figure 5.5 were isolated from *Teucrium davaeanum* by Talag, A. and Khaled Abdelhady Abdelshafeek (2006). Also compound **6** a phenyl propanoide glycoside which isolated from *T. davaeanum* (section 4.2.2) as a major constituent as shown from TLC plates are a classes of phenolic compounds.



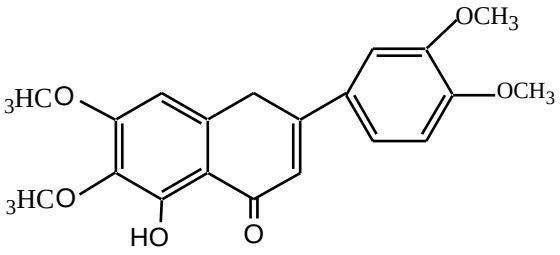
3,7,3`,4` tetra-methoxy, 5-hydroxy flavone.



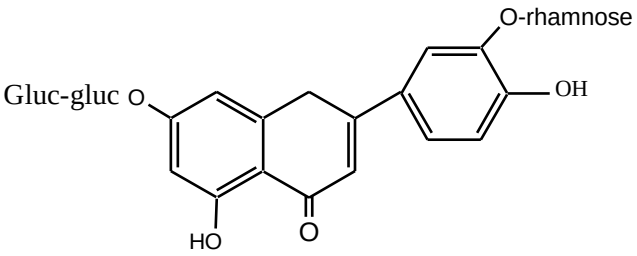
5,7,3`,4` tetra-hydroxy flavone (luteolin).



3.5` dimethyl myricetin.



5-hydroxy, 3`,4`,6,7-tatra-methoxy flavone,



Luteolin-7-O-gluco-glucosyl-3`-o-rhamnoside.

Figure 5.5 Structure of flavonoid compounds isolated from *T. davaeanum*.

5.2 Measurement of the antioxidant activity

5.2.1 Introduction

Screening for free radical scavenging activity of the two dried plant materials and *S. europaea* and *T. davaeanum* were according to ABTS radical activity. The plants extracts were prepared in 50% ethanol (10g/100ml). The antioxidant activity was measured by $ABTS^{\cdot+}$ (2.2`-azinobis(3-ethylbenzothioazoline-6-sulfonic acid) diammonium salt radical scavenging assay and determined as the concentration required for the 50 % inhibition of the radicals (IC_{50}). There are five methods to measure the antioxidant activity: 2.2-diphenyl-1-picrylhydrazyl(DPPH), 2,2``-azinobis(3-ethylbenzothioazoline-6-sulfonic acid) (ABTS), Ferric reducing antioxidant potential (FRAP), superoxide dismutase (SOD) and oxygen radical absorbance capacity (ORAC) Dudonne *et al*., (2009)

The ABTS radical solution was prepared by reacting ABTS with potassium persulfate for 6-12 hours in dark at room temperature. Potassium persulfate acts as oxidizing agent. When measuring antioxidant activity two factors should be considered; the length of reaction between oxidant and plant extract and the concentration of extract. The reaction mechanisms includes the immediate formation of the ABTS radical monocation with intermediate radical, secondly it is a decolourization assay , so the radical cation is formed before addition of antioxidant instead of formation of the radical after adding of the antioxidants.

5.2.2 Materials

UV spectrophotometer (JENWAY 6305) were from Sigma, volumetric pipets (5-50 µl, 100-1000 µl and 5 ml) (Genex Beta) were from Sigma,

volumetric flasks (10, 50, 100 and 1000) ml, cuvettes 1 ml size, Aluminium foil, para-film and gloves all were from Life Technologies. Trolox (Hoffman-La Roche) (6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid; Aldrich chemical was used as an antioxidant standard from Aldrich, $ABTS^{\cdot+}$ (2.2`-azinobis(3-ethylbenzothioazoline-6-sulfonic acid) was obtained from Sigma, Mono-sodium hydrogen phosphate NaH_2PO_4 was obtained from Sigma, Di-sodium hydrogen phosphate Na_2HPO_4 was obtained from Sigma, Analytical grade ethanol from Aldrich, Sodium chloride was obtained from Sigma, Potassium per sulphate was obtained from sigma.

5.2.3 Extraction method

Determination of the total antioxidant activity was also carried out according to Folin Ciocalteu's method. The same extraction method mentioned in section 2.10.2 but the weight of plant material was 10 g instead of 15 g.

5.2.4 Preparation of reagents

The $ABTS^{\cdot+}$ (2.2`-azinobis(3-ethylbenzothioazoline-6-sulfonicacid) was prepared by mixing 0.0481g of ABTS and 0.00825 g of potassium persulfate, dissolve each in 5 ml of distilled H_2O, combine together and then make up to exactly 50 ml with distilled water in volumetric flask. Trollex (6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid) several concentrations (0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 2 and 3 Mm) were made in analytical grade ethanol 50 %. Phosphate buffered saline (PBS) solution was made by mixing 81 ml of di-sodium hydrogen phosphate Na_2HPO_4 with 19 ml mono- sodium hydrogen phosphate NaH_2PO_4 and 0.9 g of sodium chloride. Di-sodium hydrogen phosphate Na_2HPO_4 was made by dissolving 0.709 g of it in 1 L

distilled water; while mono-sodium hydrogen phosphate NaH_2PO_4 was made by dissolving 0.689 g of it in 1 L distilled water.

5.2.5 Procedure of experimental work

Trolox several concentrations (0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 2 and 3 Mm) were made in analytical grade ethanol 50 %. The molar concentration of Trolox 1M = 250 g/L, thus, 1mM = 0.250 mg /ml, the other concentrations were made from this concentration.

The first dilution was made by dissolving 1 ml of stock solution (100 mg/ml) into 25 ml of 50% ethanol; the new concentration is 4 mg/ml. From the first diluted solution (4 mg/ml) volumes of 1, 2, 3 and 5 ml were pipetted into 10 ml volumetric flasks separately, to make a concentrations of (0.4, 0.8, 1.2 and 2) mg/ml.

5.2.6 Results and discussion

Several concentrations of Trolox were made in analytical grade ethanol 50 % (0.8, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1) Mm 2 ml each. The spectrophotometer device was blank with 1 ml PBS solution. A permanent control solution was prepared by mixing 1 ml of diluted $ABTS^{\cdot+}$ solution (50µl $ABTS^{\cdot+}$ in 1 ml PBS) with 40 µl of carrier solution 50% ethanol. The absorbance at 734 nm after 1 minute was recorded, the cuvette was kept in the dark and the absorbance was recorded again after each reading for the test solution. Figure 5.12 is shown standard curve of Trolox. The test solution was prepared by mixing 1 ml of diluted $ABTS^{\cdot+}$ solution (50µl $ABTS^{\cdot+}$ in 1 ml PBS) with several concentrations of Trolox (0.8, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1) Mm 40 µl separately. The absorbance at 734 nm was recorded each time after 1 minute. For each concentration the absorbance was recorded in triplicate.

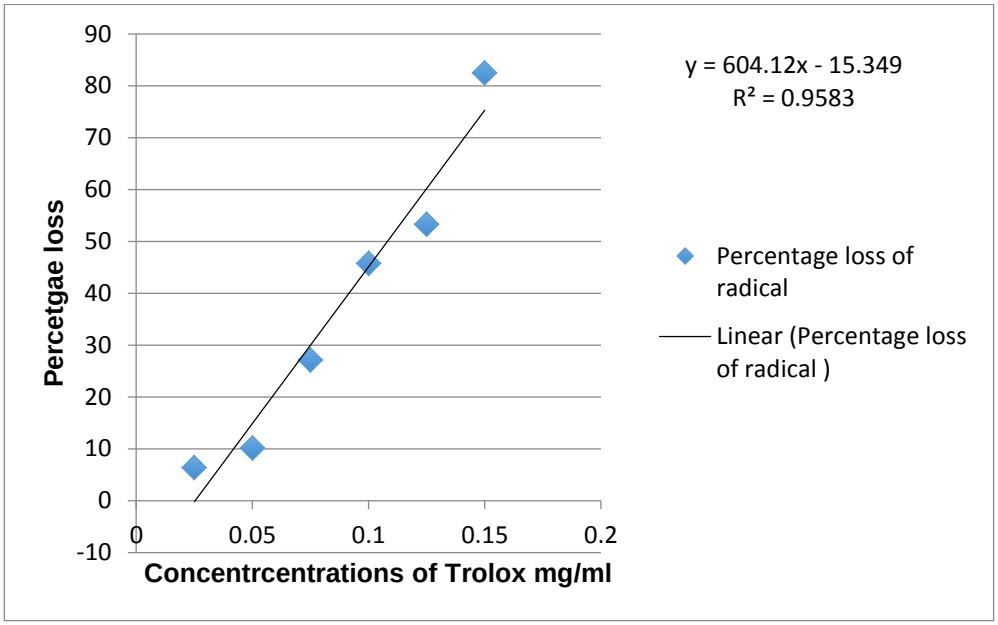


Figure 5.6 Standard curve of Trolox.

S. europaea extract was prepared by mixing 10 g of small cutting plant material into 100 ml of 50% ethanol and sonicated for 20 minutes then centrifuged at 1000 rpm for 15 minutes. The plant extract was filtered using whatman No 3 filter paper and covered with N_2 gas (stock solution 100 mg/ml). The *T. davaeanum* extract was prepared within the same procedure.

Several concentrations of S*. europaea* extract per solid plant material were made by dissolving 1 ml of stock ethanol solution into 25 ml of 50% ethanol (4 mg/ml), second dilution of *S. europaea* ethanol extract was prepared by pipetting (1, 2, 3 and 5) ml of first diluted solution (4 mg/ml) into 10 ml volumetric flasks and completed to volume with distilled water (0.4, 0.8, 1.2 and 2 mg/ml) respectively. 8 mg/ml was prepared by dissolving 1ml of stock solution into 12.5 ml ethanol (50%). The absorbance was recorded for each concentration. Figure 5.13 is shown percentage loss of radical by *S. europaea* extract.

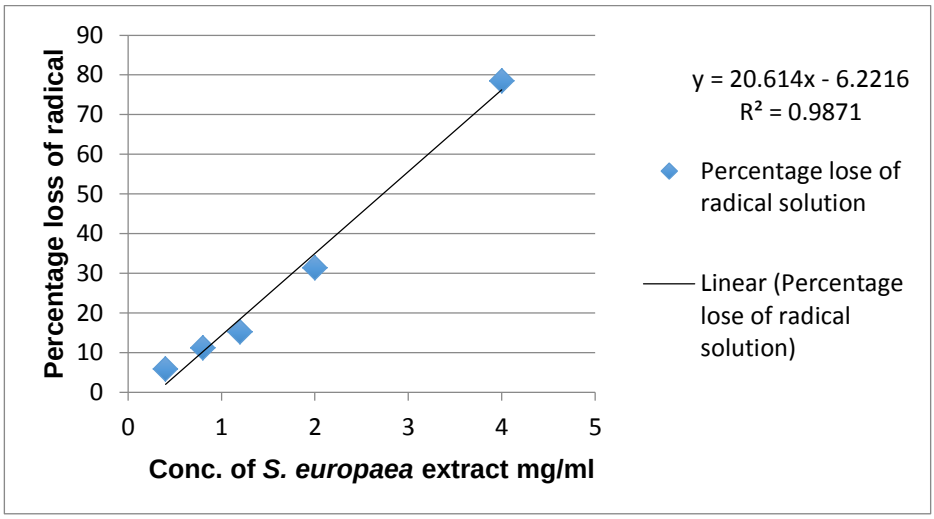


Figure 5.7 Percentage radical scavenging by *S. europaea* extract.

A total of 1ml of *T. davaeanum* extract was diluted by the same way used for *S. europaea.* The absorbance was measured for each concentration. Figure 5.14 is shown percentage loss radical by *T. davaeanum* extract.

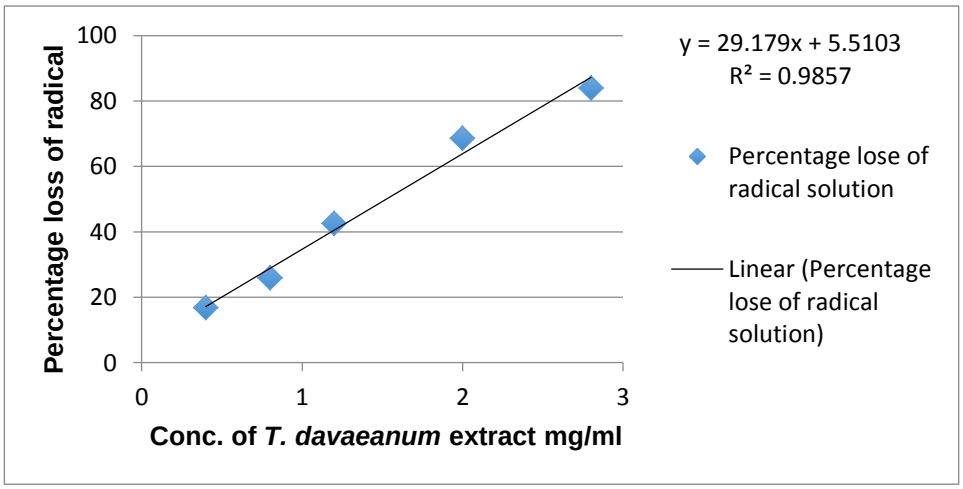


Figure 5.8 Percentage radical scavenging by *T. davaeanum* extract.

The SC equation of Trolox standard curve was used to calculate the antioxidant capacity of *S. europaea* and *T. davaeanum* the results is shown in tables 5.13 and 5.14 respectively

Table 5.1 Antioxidant capacity of *S. europaea* according to Trolox equivalent.

% loss of radical by *S. europaea* (y)	**Mg/ml equivalents to Trolox x = (y+15.487)/605.48**	**Conversion factor for 1 mg equivalent of extract (1/conc.)**	**Mg Trolox equivalent per mg of extract**
5.954	0.35096388	2.5	0.0877
11.165	0.04387	1.25	0.0548
15.182	0.050531043	0.83	0.0421
31.362	0.07732273	0.5	0.0386
78.496	0.155340191	0.25	0.0388
			Average = 0.052=5% SD = 0.02

Table 5.2 Antioxidant capacity of *T. davaeanum* according to Trolox equivalent.

% loss of radical by *T. davaeanum* (y)	**Mg/ml equivalents to Trolox x = (y+15.487)/605.48**	**Conversion factor for 1 mg equivalent of extract (1/conc.)**	**Mg Trolox equivalent per mg of extract**
16.868	0.0532	2.5	0.132
25.854	0.0682	1.25	0.085
42.504	0.0957	0.83	0.079
68.562	0.1389	0.5	0.0694
83.913	0.164	0.357	0.0586
			Average = 0.085 = 8.5% SD = 0.028

Total polyphenol contents of dried plant material of *S. europaea L* was 4.956 mg in 100 mg dried plant material. For *T. davaeanum* leaves and flowers was 1.20 mg in 100 mg dried plant material and the stem contained 0.65 mg in 100 mg dried plant material. *S. europaea* has approximately 5% antioxidant capacity of Trolox, while *T. davaeanum* has approximately 8 % antioxidant capacity of Trolox. Table 5.3 comparison the values of both total phenol content and antioxidant activity of *Sanicula europaea* and *Teucrium davaeanum* with the other plants.

Table 5.3 Comparison of total phenol content of *S. europaea* and *T. davaeanum* with other plants.

Plant	**Total phenol content**	**Trolox equivalent**	**Reference**
Sanicula europaea	4.956 mg/100 mg	5	-
Teucrium davaeanum	1.20 mg /100 mg	8	-
Yellow tea *Green* tea *Black* tea	4.116±0.113 g GAE/kg 2.262±0.113 g GAE /kg 1.139±0.064 g GAE /kg	29.345±0.124 20.738 ± 0.171 16.356 ± 0.314	(Kopjar et al., 2015).
Wissadula periplocifolia *Sidastrum micranthum* *Sidarhombilfolia* *Herissantia crispa*	260.46±5.74 mg GAE/g 177.44±16.21 mg GAE/g 88.31 ± 2.66 mg GAE/g 87.07 ± 0.50 mg GAE/g	45.71 ±0.07 2.267 ±0.377 20.58 ± 0.27 83.35 ± 0.08	(Fernandes de Oliveira et al., 2012)
Quercus robur *Pinus maritime* *Cinnamomum zeylanicum*	397.03 ± 0.05 mg GAE/g 363.02 ±0.02 mg GAE/g 309.23 ±0.05 mg GAE/g	99.80 ±0.07 83.68 ± 0.80 64.88 ±3.74	(Dudonne et al., 2009)

It can be concluded that both species have a medium amount of total phenol content and antioxidant activity.

5.3 Cytotoxicity assay

5.3.1 Introduction

HeLa cell line was the first continuous cancer cell line isolated from the aggressive glandular cervical cancer of a young woman (Henrietta Lacks) over 100 years ago. The cytotoxic activity of four compounds isolated from the crude glycosides extract of *T. davaeanum* was determined using this cell line. The cytotoxic activity was kindly carried out by Dr Amos Fatokun University of Bradfrord, according to the method published in (Fatokun et al., 2013).

5.3.2 Materials

Minimum Essential Medium (MEM) was purchased from Life technologies. Foetal Bovine Serum (FBS) was from Sigma. Phosphate Buffered Saline (PBS) was from Sigma. Stocks of L-glutamine and antibiotics were from Life Technologies. Trypsin/EDTA (TrypLE™ used) from Life Technologies. Alamar blue (AB) from Life Technologies. Molecular Device's FlexStation 3. Incubator was from Life Technologies. 96-well black flat-bottom plates (micro-clear), plastic ware was from Life Technologies.

5.3.3 Procedure of experimental work

The growth medium used was Minimum Essential Medium (MEM) supplemented with 10% Foetal Bovine Serum (FBS), 2 mM L-glutamine, 1% antibiotic-antimycotic solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of Fungizone® Antimycotic) and 1% non-essential amino acids (NEAA). Cultures were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2. Procedures were carried out on cultured cells under aseptic conditions (Class II flow cabinet). Growth

medium and other solutions were warmed to 37^{o}C before they were used on cultures.

The growth medium was aspirated from the cultures growing in a 75cm^{2} flask and the cultures were washed with PBS. PBS was aspirated and sufficient volume of TrypLE was added to cover the surface of the dish. After 2-3 minutes, TrypLE solution was removed and the flask was returned to the incubator for 2-3 minutes, after which it was taken out of the incubator and the trypsinised cultures flooded with the growth medium. The growth medium was pipetted up and down and squirted around the surface of the flask to remove the adherent cells. The cell suspension was transferred into a 30 ml tube and cell density was determined using the haemocytometer. Cell density was then adjusted to the desired density of 1×10^{5} cells/ml. A 100 µl aliquot of the suspension was then seeded into each well of the required number of 96-well plates and the plates were incubated overnight.

5.3.4 Cell viability assay

Dilutions (concentrations) of each test compound were prepared from their stock solutions (made up in DMSO) by diluting with MEM. The growth medium on the cultures was aspirated and the cultures were incubated for 48 h with the prepared concentrations of the compounds, making sure that the final DMSO concentration did not exceed 0.1% (which is not toxic to the cells). Each concentration was added in triplicate. Appropriate controls (treated with growth medium alone) were included. After the 48 h incubation, the viability dye Alamar Blue (AB) was added to the cultures at 10% v/v and plates were further incubated for 3 h at 37^{o}C. AB is usually added in a single step, and the extent of its reduction is indicated by a colour change (blue to red), which is directly proportional to the viability of the cell population.

After 3 hours incubation with AB, plates were taken out of the incubator and left at room temperature for 20 min and the dye's fluorescence was determined on Molecular Device's Flexstation 3, using, according to the manufacturer's protocol, an excitation of 530 nm (544 nm used) and an emission of 590 nm.

5.3.5 Results and discussion

Data were analysed by comparing the average fluorescence value of the triplicate wells for each concentration with the average fluorescence value of the triplicate control wells. Control average was then set as 100%, to which each of the rest averages was normalised. Statistical analysis was done using GraphPad Prism (GraphPad Software, CA, USA). Inhibition of cell growth (IC_{50}) by glycosides 6, 7, 8 and 10 isolated from *T davaeanum* against Hela cells line (The results are a mine of four separate determinations) is shown in table 5.15, Figure 5.15.

Table 5.4 Inhibition of cell growth by compounds 6,7,8 and 10.

Compound	**Conc. µg/ml (IC_{50})**			
	12.5	25	50	100
6	99.5	97.5	95.9	90.3
8	99.1	98.5	97.2	95.5
10	93.8	76.5	15.2	6.2
7	99.8	98.2	96.3	93.1

Study of cytotoxic activity of four glycoside compounds isolated from *T. davaeanum* crude glycosides extract was kindly carried out by Dr. Fatokun according to the method published by Fatokun *et al* (2013). The results

showed that saponin compound **10** reduce Hela cell viability (IC_{50}) at concentration 50 µg ($P<0.001$). 10 has a moderate cytotoxicity compared with other saponins which have a high cytotoxicity such as, saponin compounds isolated from *Sea Cucumber Holothuria ForskalII*. The IC_{50} of Holothurinoside A (4) 0.86 µg and Holothurinoside C 0.47 µg (Rodriguez et al., 1991). The inhibition % at 50 µg/ml of two saponin compounds isolated from Brodiaea California were 12.8 and 54.7 of (25S)-spirost-5-ene-1β,3β-diol[(25S)-ruscogenin]-1- *O*- β - D- glucopyranosyl- (1→3) -*O*- α- L-rhamnopyranosyl-(1→3) - β- D- glucopyranoside] (saponin A) and(25S)-ruscogenin-1-*O*-{*O*-β-D-glucopyranosyl - (1→3) -*O*- L-rhamnopyranosyl-(1→2)-*O*-[β-D- xylopyranosyl-(1→3)]- β -D-glucopyranoside} (saponin B) respectively (Mimaki et al., 1995).

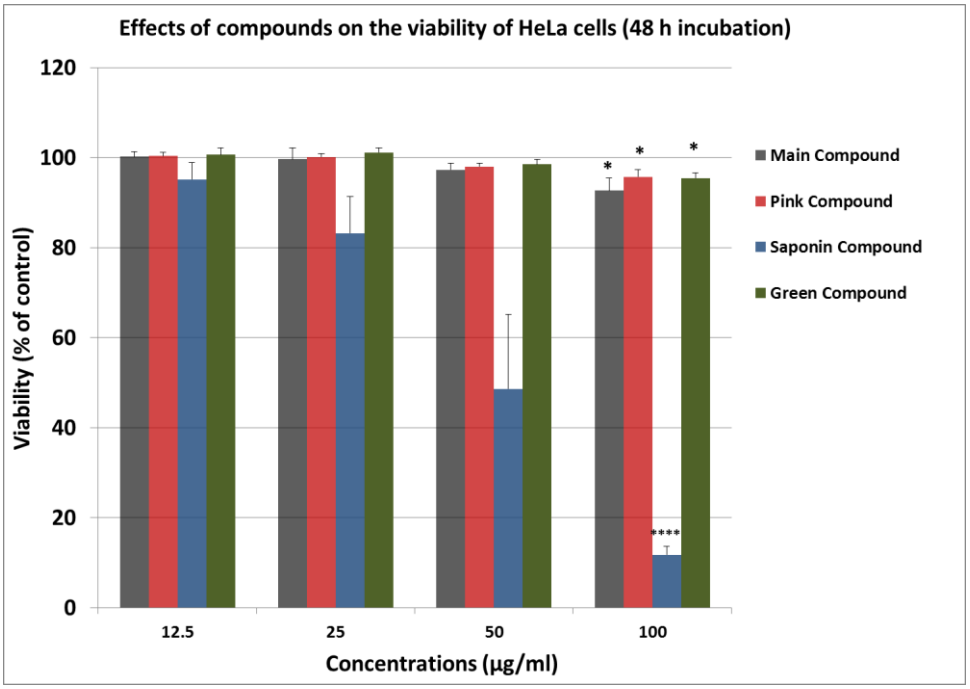
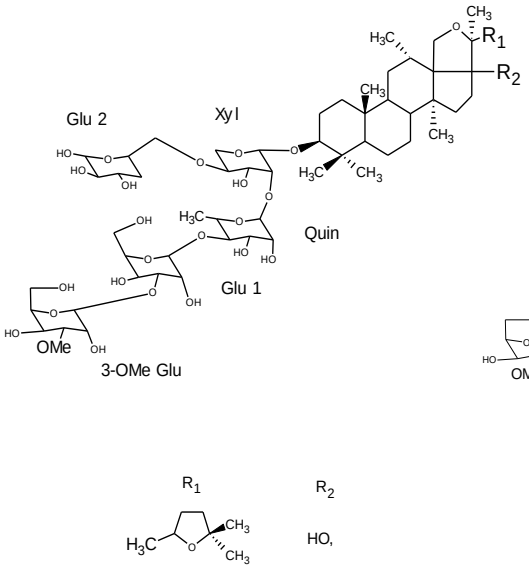
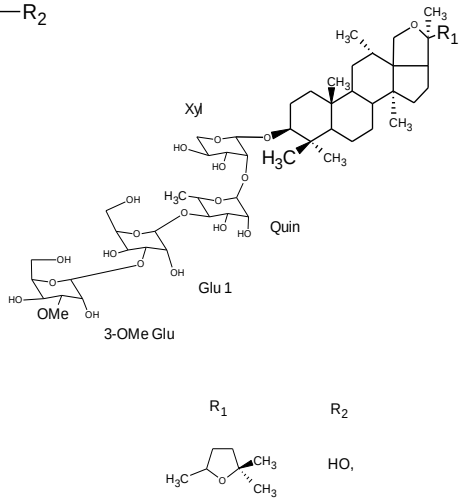


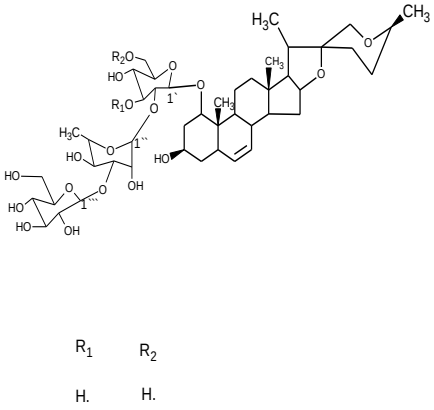
Figure 5.9 Effects of compounds from *T. davaeanum* on the viability of Hela cells9. The gray colour is refer to compound 6, red (8), blue (10) and green (7).



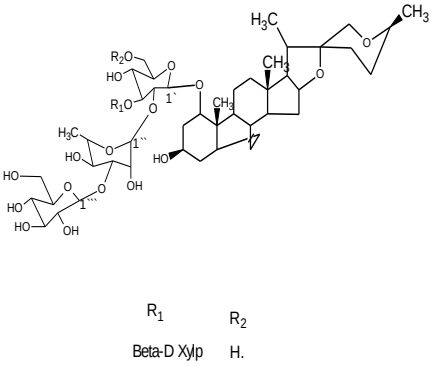
Holothurinoside A



Holothurinoside B



Saponin A



Saponin B

Figure 5.10 Some saponins possessing cytotoxic activity.

5.3.6 Cytotoxic mechanisms of saponins

Some saponins are potential anticancer agents, with diverse mechanism of action. Their cytotoxic activity could be related to either apoptosis inducement or non-apoptotic cell death stimulation. A number of processes resulting in cell death but involving different mechanisms of action are recognised, including stimulation of autophagic cell death, decrease in NO production in cells, cytoskeleton integrity disassembly, angiogenesis or metastasis inhibition and cell cycle arrest. The cytotoxic mechanism of some saponins from natural resources will be discussed in the following section. The immunosuppressive activity of a triterpene, saikosaponin A from *Bupleurum falcatum* on concanavalin A (Con A) stimulated mouse $SD3^+$ T cells, isolated from the lymph node of BALB/c mice, was examined, as well as the underlying mechanism of the action. This compound had significant antiproliferative activity, and also potentially inhibited the activation of the cells, in a concentration-dependent manner. Furthermore, saikosaponin A potently suppressed the production of IL-2, IFN-ɤ and TNF-α in T cells stimulated by Con A. The G_0/G_1 arrest of the activated T cells by saikosaponin A was also observed, and the activity was due to down-regulation of CDK6 and cyclin D3 proteins and up-regulation of $P27^{kip}$. The apoptosis of the activated T cells, in comparison to the non-activated cells, was induced by the compound in a dose-dependent manner, probably through the mitochondrial pathway (Sun et al., 2009) .

The anticancer activities of ardisiacrispin (A + B), a mixture (fixed proportion 2:1) of triterpene saponins ardisiacrispin A and B from *Ardisia crenata*, toward a number of human cancer cell lines were studied. The highest antiproliferative activity of the tested substance was evaluated on human hepatoma Bel-7402 cells. Furthermore, the compound induced apoptosis in Bel-7402 cells, which was observed by the changes of the mitochondrial membrane depolarization, membrane permeability

enhancement and nuclear condensation, and the effects were dose-dependent (Li *et al*., 2008).

The cytotoxic activity of a triterpene saponin, tubeimoside 1 (TBMS1) from *Bolbostemma paniculatum* was tested on Hela cells. In the cells treated with compound, profound protein alterations were observed, suggesting an apoptotic process, with the destruction of mitochondria and endoplasmic reticulum. TBMS induced the depletion of mitochondrial transmembrane potential, causing the activation of caspase-dependent apoptotic cell death. Moreover, the increased expression of GADD153/CHOP transcription factor, associated with growth arrest and apoptosis in the event of prolonged ER stress, was detected. The results of the study indicate that the cytotoxic effect of TBMS1 operates through the mitochondrial, but also ER stress cell death pathways (Xu *et al*., 2009).

5.4 Conclusion

The present thesis includes a study of the chemical constituents of two species *Sanicula europaea L*. belonging to (Apiaceae) and *Teucrium davaeanum L*. belonging to (Labiatae). Both species were traditionally used in treatment of wounds. *S. europaea* and *T. davaeanum* were subjected to phytochemical investigation concerning their lipid fraction as well as total crude glycosides extracts prepared from butanol fraction.

The GC-MS analysis of fatty alcohols fraction of *S. europaea* revealed the presence of heptacosane (43.02 %) as a main component.

The unsaponifiable fraction of *S. europaea* was investigated by GC-MS. The results showed that the unsaponifiable fraction consists mainly from a mixture of β-selinene (76.09%), caryophyllene oxide (4.20%) and pentadecanone (2.23%).

The study of total fatty acids of *S. europaea* was achieved via GC-MS analysis of their methyl esters. The obtained results revealed the presence of 7-Hexadecanoic acid, methyl ester (2.23%), nonanedioic acid, dimethylester (1.72%), cyclopropane butanoic cid (0.43%), Caffeine (7.93%), isopropyl myristate (1.05%), 1,2,Benzene dicarboxylic acid, bis (2-methyl propyl) ester (1.98 %), 9, 12, octadecatrienoic acid (17.47%), oleic acid, 3-(octadecyloxy) propyl ester (1.21%), heptadecanoic acid, 16 methyl –methylester (2.68%).

Crude glycoside extracts of *S. europaea* and *T. davaeanum* were prepared from the methanol extracts (80%) by treating the concentrated alcoholic extract with water followed by extraction with organic solvents, hexane, chloroform, ethyl acetate and butanol. The dried butanol extract was then dissolved in methanol, and dropped slowly into diethyl ether. The whitish precipitate obtained from the above procedure was filtered using a sintered glass Buchner funnel. The precipitate was dried under vacuum for approximately 18 hours.

Purification of crude glycosides extract of *S. europaea* was partially achieved using open column and preparative TLC chromatography, different adsorbents including normal or reverse phase silica gel and Sephadex LH-20 were used. Preparative TLC was successful in purification of one saponin compound **4** (saniculaside N).

High performance liquid chromatography technique HPLC was used to purify the saponin fractions of *S. europaea.* Analytical HPLC (isocratic method) was successful in establishment of isolation parameters of saponin fractions. The best two mobile phases for fractionation of saponins fraction of *S. europaea* on analytical HPLC column connected to normal phase silica gel column were CH_3CN:H_2O:Isopropanol, 35:60:5, flow rate 3 ml/min, and $CHCl_3$:MeOH:H_2O, 60:45:5, flow rate 1 ml/min figure(5) because with decreased the flow rate, the saponins will have enough time to bind to silica gel, and the saponins will separated into

several peaks instead of single peak. Therefore the less polar compounds will eluted from the column first followed by more polar compounds.

The flow rate has a significant effect on purification of saponins fraction of *S. europa*ea on normal phase silica gel column, as decreasing the flow rate improved the separation of the peaks.

Purification of saponin fraction B was successfully achieved using preparative high performance liquid chromatography technique (PHPLC) using a normal phase silica gel column. Four different gradient systems were used to purify the saponins fraction B (the purest sample) via preparative HPLC system connected to normal phase silica gel column. Four methods were applied, method three was successful in separation of glycoside compound named saniculaside N (4) (fraction eluted at 20 minute).

Four known compounds were isolated from the 80% methanolic extract of *S. europaea* named bis (2-ethylhexyl) phthalate (**1**), palmitic acid (**2**), rosmarinic acid (**3**), saniculoside N (**4**). A mixture of sugars (**5**) composed from sucrose, glucose and fructose was also isolated from *S. europaea*. Compounds **1**, **2** and **5** were isolated for the first time from this species. Identification of the isolated compounds was achieved through spectroscopic measurements including 1-D(^{1}H &^{13}C NMR), 2-D(HMQC, HMBC and cosy) and mass spectrometry ES-, ES+ in addition to accurate mass measurement. Figure 5.17 structure of compounds isolated from *S. europaea*.

Several experiments were carried out in order to determine the origin of **1** in *S. europaea* (section 3.4.4). The results revealed that **1** is a constituent of *S. europaea* and not a contaminant from the solvents or plastic equipment. Thus further work needs to be carried out to confirm whether it is synthesized in the plant itself or absorbed by the plant from the atmosphere or the soil.

The GC-MS analysis of fatty alcohols fraction of *T. davaeanum* showed the presence of three hydrocarbons named heptacosane (4.5%), octacosane (35.4) and tetratetracontane (51.0%).

The unsaponifiable fraction of *T. davaeanum* showed the presence of caryophyllene (0.96%), spathulenol (4.41%), caryophyllene oxide (3.66%), teu-Muurolol (3.34%), α-Bisabololoxide B(11.12%), α – bisabolol (4.57%), 2-pentadecanone (1.66%) and phytol (6.79%).

The study of total fatty acids of *T. davaeanum* was carried out via GC-MS analysis of their methyl esters. The obtained results revealed the presence of decanoic acid methyl ester (0.35%), 14-octadecenal (1.07%), dimethyl phthalate (3.0%), dodecanoic acid methylester (8.27%), cyclopropaneoctanoic acid (1.03%), methyl tetradecanoate (7.12%) and hexadecanoic acid methyl ester (14.56%).

Purification of the crude glycosides extract of *T. davaeanum* was effected by applying column chromatography using different adsorbants including normal or reverse phase silica gel or Sephadex LH-20. Moreover, further purification was achieved using preparative TLC as well as Sephadex LH-20 column chromatography.

Five compounds were isolated from the crude glycosides extract of *T. davaeanum*. Two among them may be a novel; **6** is phenylethanoid glycoside for which the name davaeanuside A is proposed and **8** is an iridoid glycoside for which the name davaeanuside B is proposed. Sucrose (**9**) was isolated for the first time from *T. davaeanum L*. In addition to two partially identified compounds, **7** is expected to be an iridoid glycoside and **10** could be a triterpene glycoside. Figure 5.18 structure of glycoside compounds isolated from *T. davaeanum*.

The total phenol content and antioxidant activity of *S. europaea* and *T. davaeanum* 50% ethanolic extracts were achieved using the Folin Ciocalteu method. The result showed that the total polyphenol content of *S. europaea L* is 4.956 mg per 100 mg dried plant material. For *T. davaeanum* leaves and flowers the phenol content was 1.20 mg per 100 mg dried plant material, and for *T. davaeanum* stem was 0.65 mg per 100 mg dried plant material.

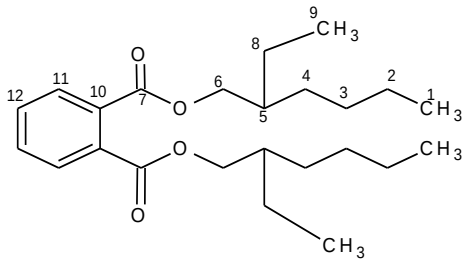
The study of the antioxidant activity of the 50 % ethanol extract of *S. europaea* and *T. davaeanum* showed that on a mg/mg basis *S. europaea* has approximately 5% antioxidant capacity of Trolox and *T. davaeanum* has approximately 8 % antioxidant capacity of Trolox. These values are moderate compared with yellow green and black tea which have antioxidant activity according to ABTS radical scavenging of 29.345 ± 0.124, 20.738 ± 0.171 and 16.356 ± 0.314 µmol/100g respectively (Kopjar et al., 2015) .

The study of cytotoxic activity (IC_{50}) of davaeanuside A (**6**), iridoid glycosides (**7**), davaeanuside B (**8**) and saponin compound (**10**) isolated from the crude glycosides extract of *T. davaeanum* revealed that saponin compound (**10**) has cytotoxicity activity (IC_{50}) against Hela cell lines at 50 µg/ml, $P < 0.001$, but the other compounds did not show activities against the tested cell lines at 100 µg/ml.

The wound healing activity of *S. europaea* could be related to presence of rosmarinic acid (**3**), because **3** (the main compound in crude glycoside extract of *S. europaea* as showed on TLC) was reported to has antinflammatory activity in many journal articles such as (Domitrović et al., 2014) (Costa et al., 2012) (Lee et al., 2008). Furthermore rosmarinic acid was mentioned to have antimicrobial activity (Bais et al., 2002); (Petersen and Simmonds, 2003) (Szabo et al., 1999). Thus the anti-inflammatory and antimicrobial activities may contribute to the wound healing activity.

In *T. davaeanum* the potentially novel compound **6** which is the main compound in crude glycosides extract (as showed on TLC) has a similar skeleton to the rosmarinic acid (main compound in *S. europaea* methanol extract) as both are phenylpropanoids, despite the fact that in rosmarinic acid the aglycone does not have a glycoside moiety, therefore the wound healing activity of both species could be related to presence of this class of compounds.

Hexadecanoic acid methyl ester (palmitic acid) showed to be the major constituents of total fatty acid methyl ester of *T. davaeanum* (Table 4.3 and Figure 4.3). This compound mentioned in treatment of wound by (Victor R. Preedy et al., 2011) . Palmitic acid was isolated from the hexane extract of *S. europaea* (section 3.4.5) and also identified in GC-MS analysis of fatty acid methyl ester of *S. europaea* (section 3.1) therefore the wound healing activity of both species could be related to the present of this compound. The unexpected finding of **1** in both plant species and the important of this as **1** is common used in plastic manufacture and are known to be toxic.



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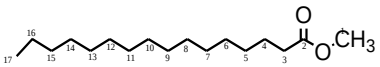

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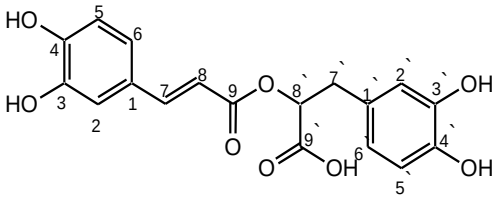

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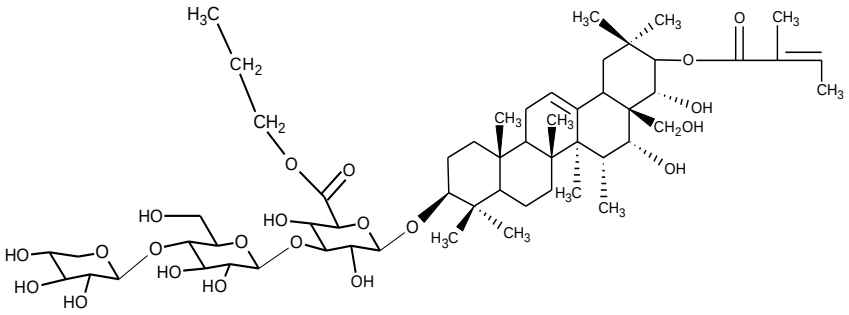

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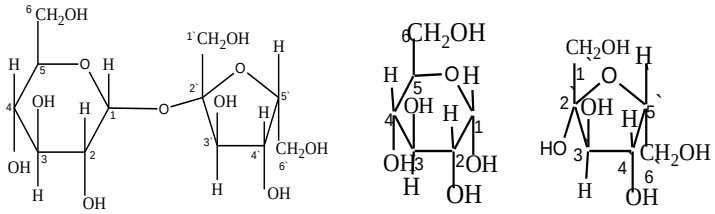

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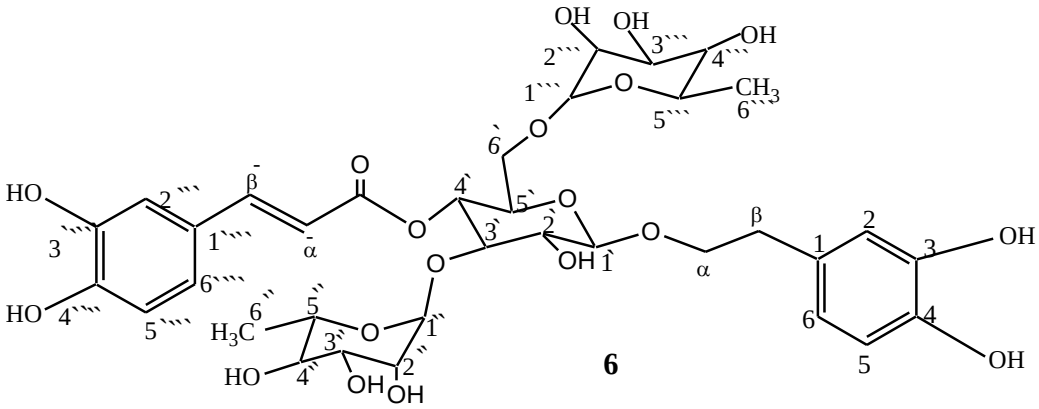
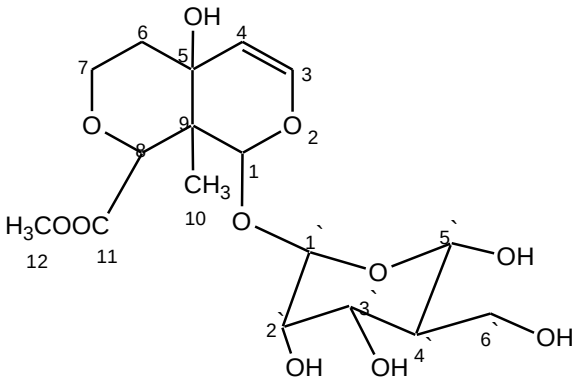
Figure 5.11 Structure of compounds isolated from *S. europaea*.



6



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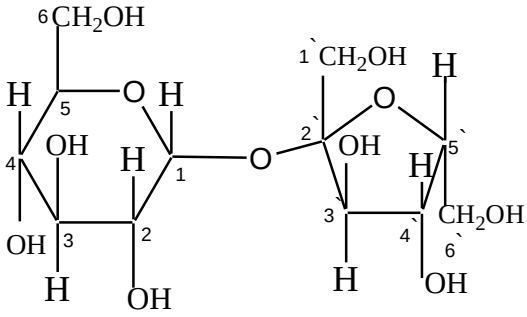

9

Figure 5.12 Structure of compounds isolated from *T. davaeanum*.

5.5 Further work

- Determine the source of **1** in *S. europaea* by extraction of a samples of the soil in which the *S. europaea* is growing (different areas) or growing of *S. europaea* in a special atmosphere which supplied it only with oxygen then carried out the extraction of a sample from this source and producing TLC analysis of the total extract and a sample of **1.**

- Collect sufficient quantity of fraction eluted at 13 minutes (major peak) from the saponin glycosides of *S. europaea* using preparative HPLC technique, method three which mentioned in section 3.5.2 and complete structure elucidation of the isolated compound using 1-D & 2-D NMR spectroscopy and mass spectrometry, also carry out the wound healing assay.

- Study the stereochemistry of **6** and **8** isolated from the crude glycosides of *T. davaeanum* using X-ray crystallography in order to have 3D structure or by nuclear overhauser effect NMR spectroscopy (NOE).

- Complete the structure elucidation of compounds **7** and **10** isolated from crude glycosides extract of *T. davaeanum* using 1-D & 2-D NMR spectroscopy.

- Evaluate the cytotoxic activity of compound **10** isolated from crude glycosides extract of *T. davaeanum* against other cell lines such as P-388, A-549 and B-16 because the saponin compound isolated from Sea *Cucumber Holothuria ForskalII* has 5 sugar unites (similar to **10** which has 6 sugar unites or more) showed cytotoxic activity against these cell lines.

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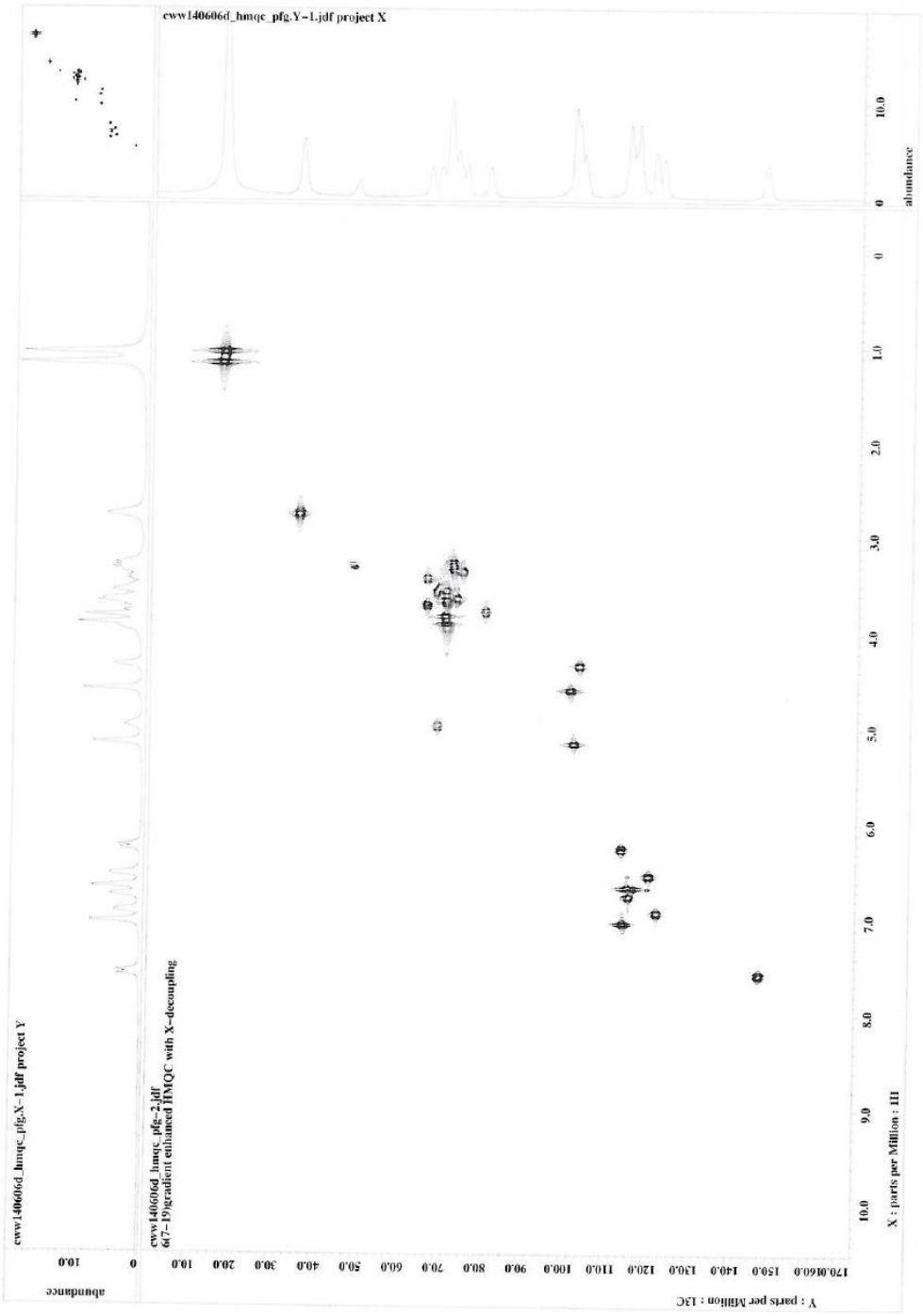
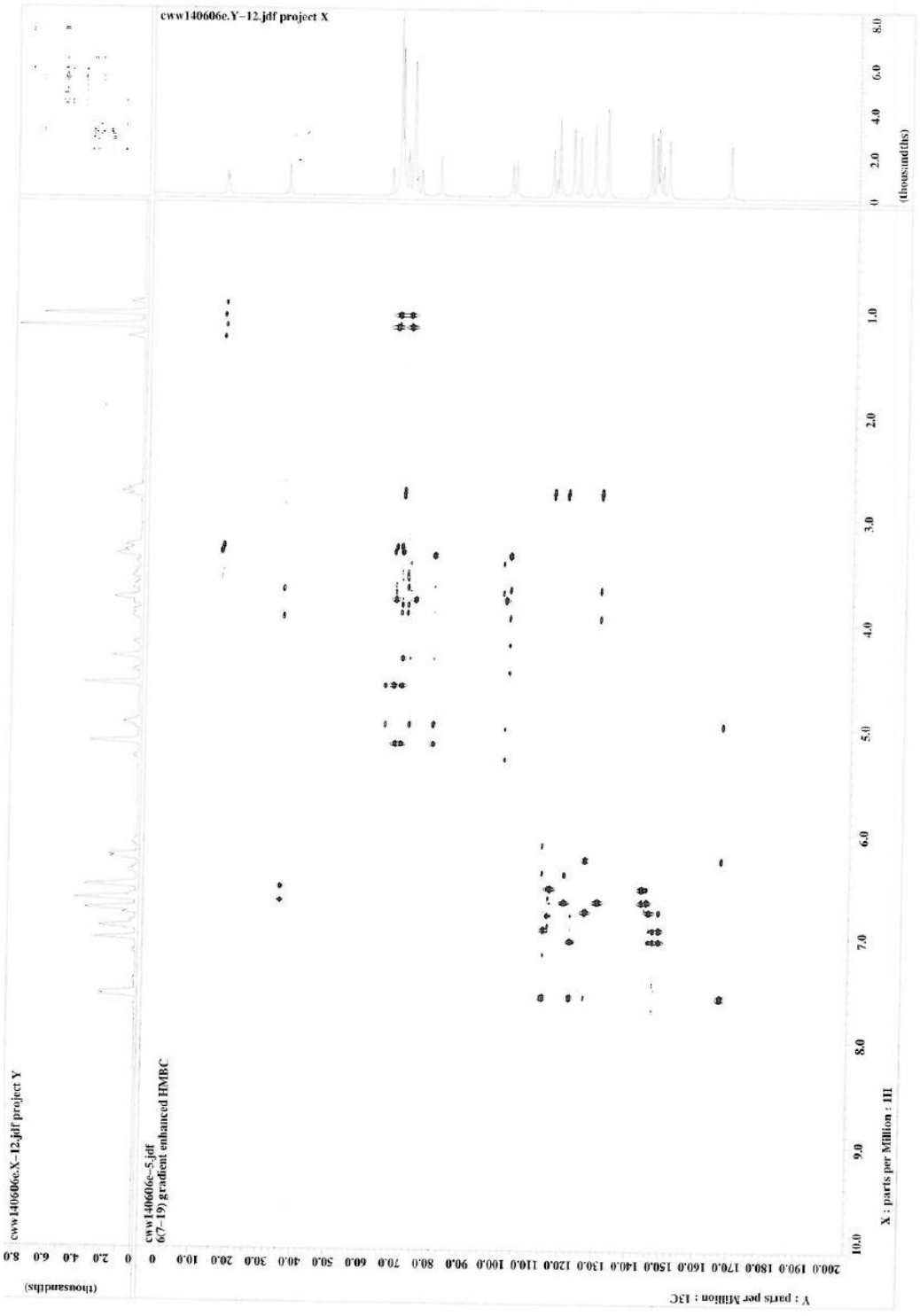
Appendices

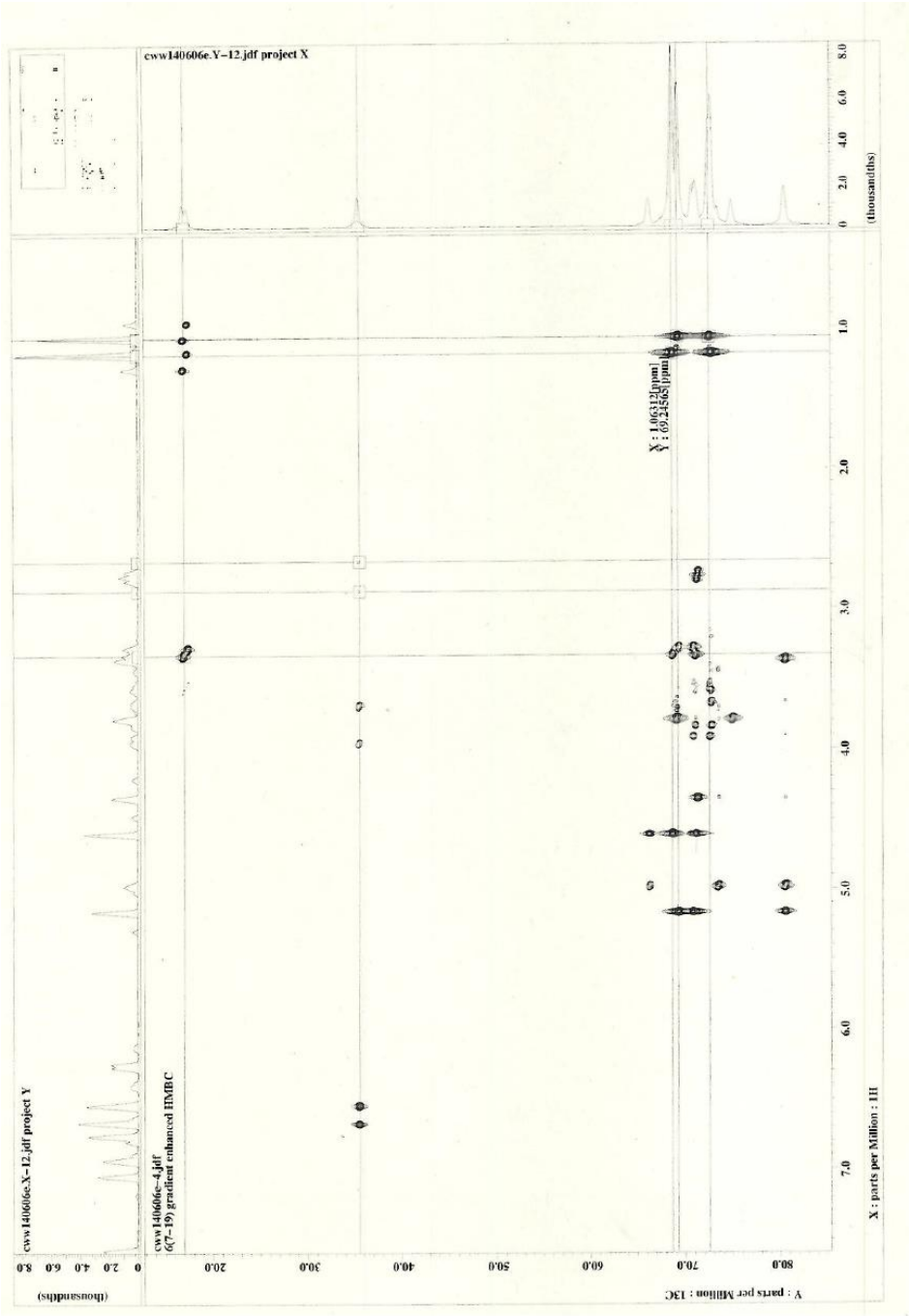
Appendice 1a HMQC spectrum of 6.



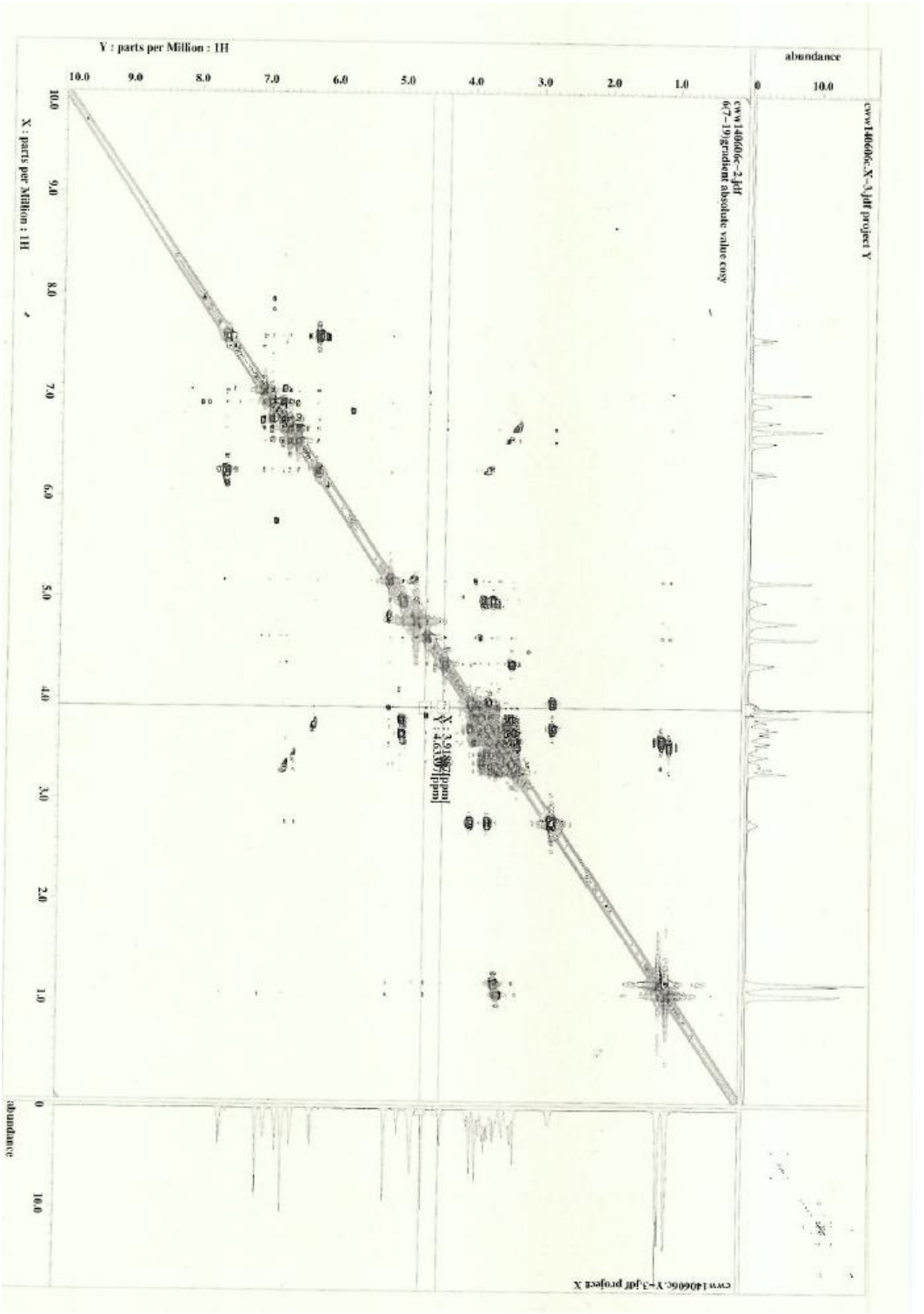
Appendice 1b HMBC spectrum of 6.



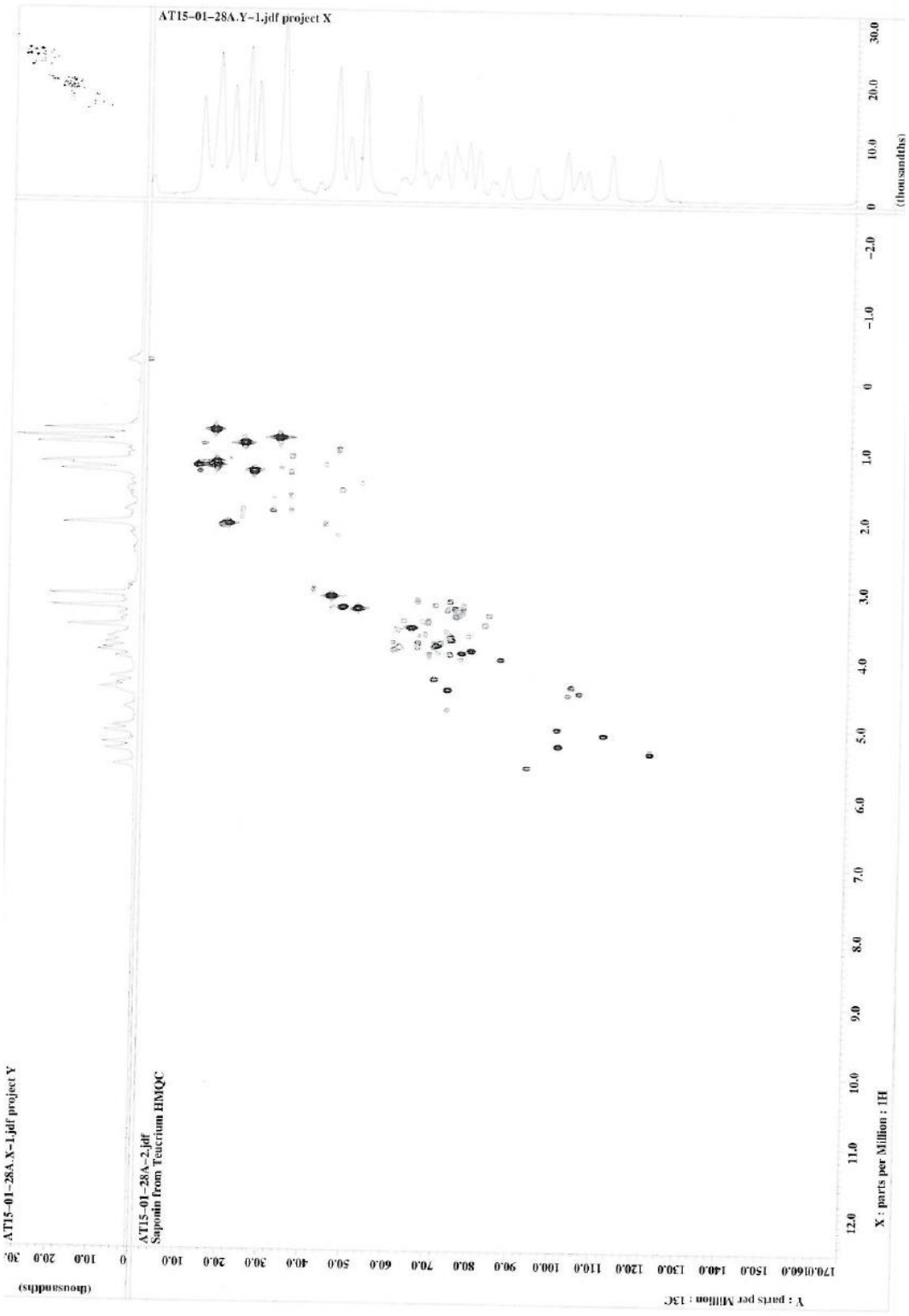
Appendice 1c Cosy spectrum of 6.



Appendice 1c Continued.



Appendice 2a HMQC spectrum of 10.



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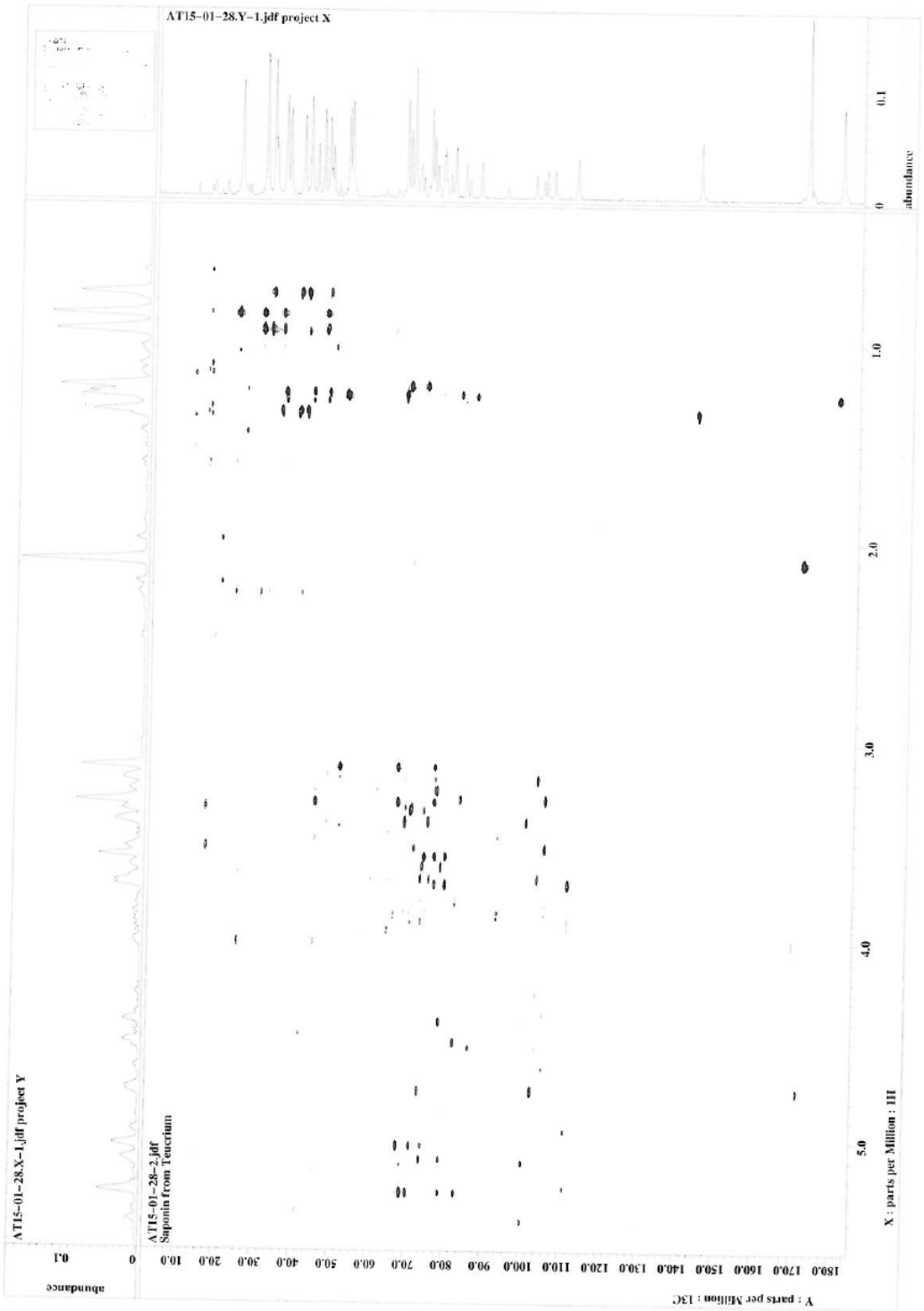

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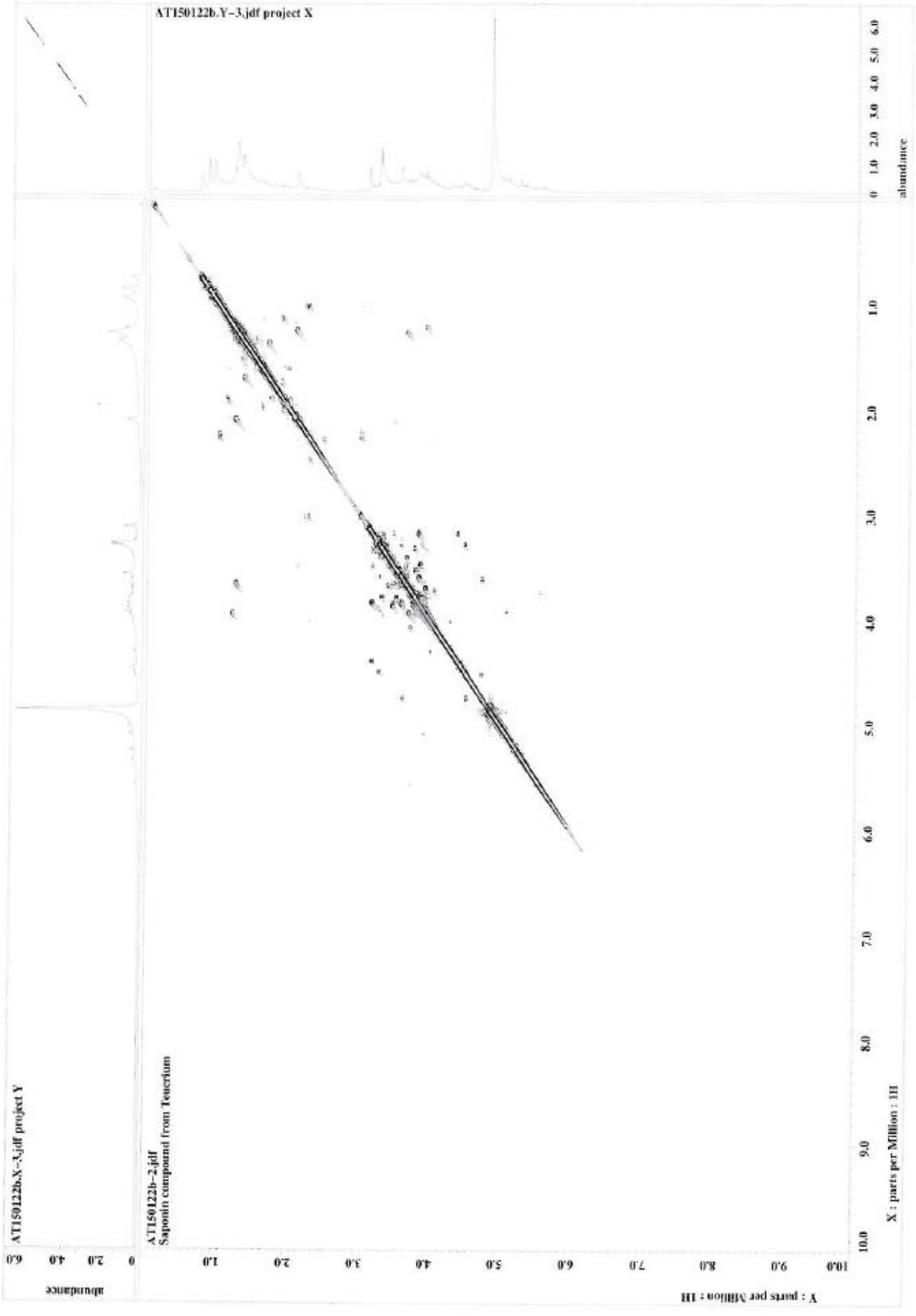
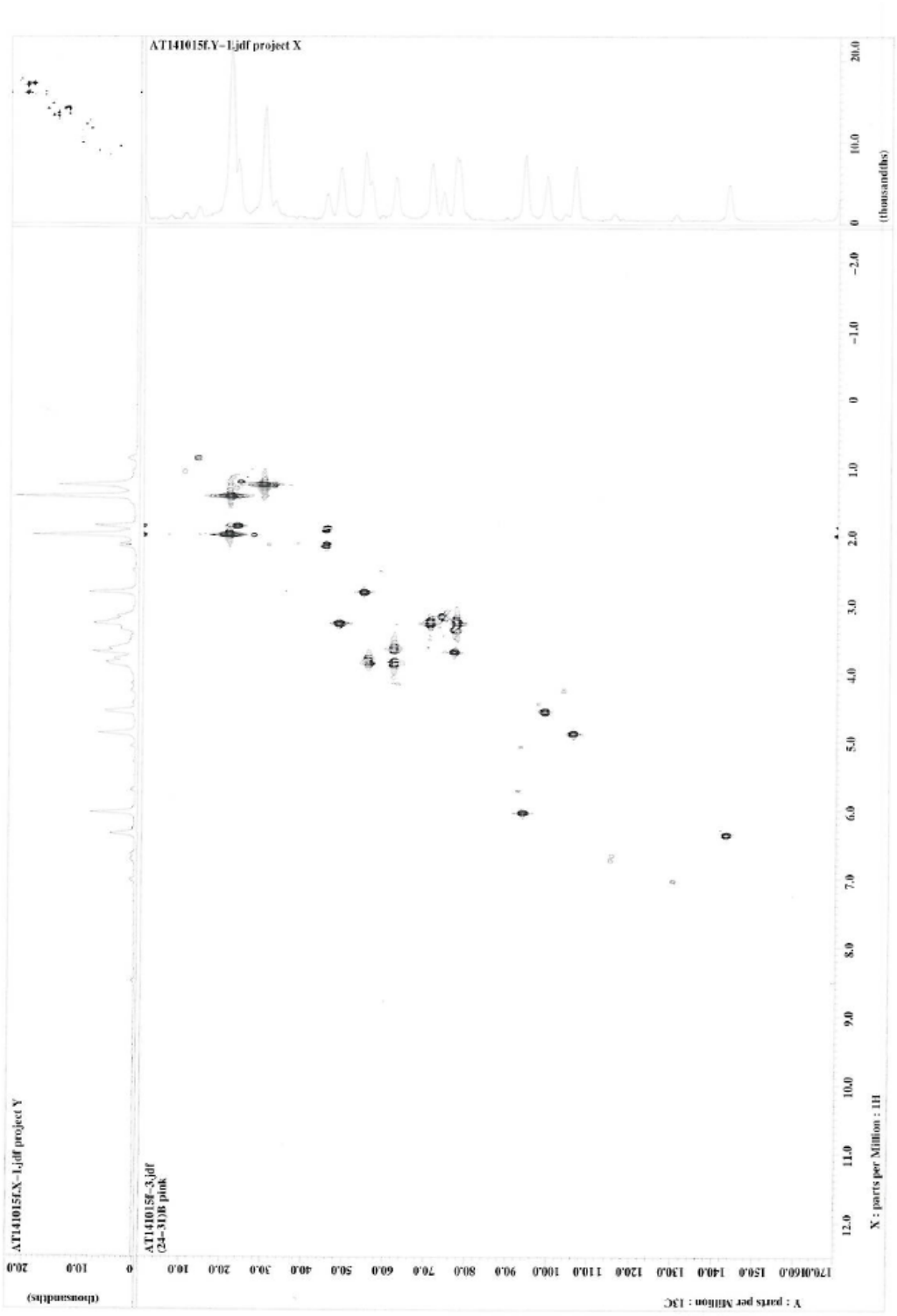


Appendice 3a HMQC spectrum of 8.



Appendice 3b HMBC spectrum of 8.